FDA Public Workshop on Next-Generation Sequencing-Based Oncology Panels

White Oak, MD February 25, 2016

Webcast address: https://collaboration.fda.gov/ngsop0216/

- **Please set phones, computers and blackberries to silent mode.**
- **Wifi can be accessed in the Great Room area using the code *guestaccess***
- **Food and beverage are available for purchase at the kiosk in the registration lobby during breaks and lunch**
- **Links to the meeting transcript and archived webcast will be posted the workshop registration website 6-8 weeks after the meeting**

Welcome!

Elizabeth Mansfield, Ph.D.
Deputy Office Director
Personalized Medicine
FDA/CDRH/OIR

Overview of Meeting Goals

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Next Generation Sequencing-Based Oncology Panels: Overview of meeting goals

FDA Public Workshop
February 25, 2016

Reena Philip, Ph.D.
Director
Division of Molecular Genetics and Pathology
OIR/CDRH/FDA

Outline

- Background
- Scope
- Hypothetical Case
- Workshop Discussion Topics
  - Potential general intended use
  - Pre-analytical and quality metric approaches
  - Analytical validation, bioinformatics, and post-approval assay modifications
  - Clinical and follow-on companion diagnostic claims

Personalized Medicine

The success of personalized medicine depends on having accurate, reproducible and clinically useful companion diagnostic tests to identify patients who can benefit from targeted therapies

Companion Diagnostics are those tests that provides information that is essential for the safe and effective use of a corresponding drug or biological product.

FDA Expectation for Companion Diagnostics

"Guidance for Industry and FDA Staff: In Vitro Companion Diagnostic Devices"

- Finalized on August 6, 2014
- Defines companion diagnostic device and various scenarios for use
- Describes FDA policies for approval and labeling
- Recommends contemporaneous regulatory approvals of the device and drug

Overview of Companion Diagnostic Validation

- Analytical validation
  - Conducted with clinical specimens from the intended use population (exception for rare mutations)
  - Analytical validation (e.g., accuracy, reproducibility, specificity, stability) obtained with attention to the clinical decision point
  - Studies are aligned with the assay technology such as accuracy for molecular assays, inter-reader agreement for IHC assays
- Clinical validation of the device is supported by the results of the drug trial when a companion diagnostic is used to test specimens and identify patients eligible for the trial.

"Follow-on" Companion Diagnostics (CDx)

- **Defining "Follow-on" CDx**
  - The same **intended use** and **therapeutic indication** as the originally-approved CDx on the market (e.g., an indication for use with Herceptin)

"Follow-On" CDx

- "Follow-on" CDx should consistently and accurately select the same intended use patient population as the originally-approved companion diagnostic devices for the indicated therapeutic drug.

- "Follow-on" CDx should demonstrate the same or comparable level of analytical and clinical performance for specific mutations in the originally-approved companion diagnostic device.

Oncology CDx assays

- A number of oncology therapeutic products have been approved with corresponding companion diagnostics.
- To date, approved companion diagnostic assays assess a single analyte or pre-specified mutations associated with therapeutic response.
- Next generation sequencing (NGS) tumor panels are increasingly employed for use in oncology applications.
- NGS technology can interrogate a patient's tumor specimen for numerous biomarkers concurrently, introducing challenges to the current companion diagnostic regulatory paradigm.

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NGS-based Oncology Panels Public Workshop

- To obtain input from external stakeholders on approaches
  - To establish analytical performance characteristics of panels that include variants intended to be used as companion diagnostics as well as other variants that may be used for alternative therapeutic management of patients who have already been considered for all appropriate therapies
  - To produce the clinical information that is needed to support follow-on companion diagnostic devices

NGS-based Oncology Panels Public Workshop

- Requesting public input on strategies for establishing performance characteristics for NGS-based oncology panels for
  - Rare variants across tumor types
  - Claims for follow-on companion diagnostic claims
  - Post-approval assay modifications

Today's Workshop

- Focus on manufacturers actively marketing NGS-based oncology panels
  - Truth in labeling (commercialization and marketing)
  - Adequate representation of panel performance for a user to decide how and when to use the panel

Scope of the Workshop

- NGS-based oncology panels for human genomic DNA/RNA
  - intended to be used as companion diagnostic devices for the clinical management of previously diagnosed oncology patients
  - Alternative therapeutic management for patients who have already been considered for all appropriate therapies

Scope of the Workshop

- Does not apply to:
  - Subjects who have not been diagnosed with cancer
    - e.g., Cancer risk assessment and standalone clinical diagnosis
  - Circulating tumor DNA testing
  - IVDMIAs using NGS inputs
  - WES or WGS
  - Assays detecting non-human sequences
  - Carrier screening for hereditary genetic disorders
  - Quality of databases

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NGS-based Oncology Panel Workflow

- Specimens – Type, collection and handling methods, preparation for DNA/RNA extraction, and storage
- Nucleic acid preparation – Extraction method and assessment of quality
- Library Preparation – Primer design, amplification, capture type, quality assessment
- Sequencing – Sequencing platform, reagents, platform validation.
- Base calling
- Alignment/mapping
- Variant calling
- Annotation/filtering/variant classification
- Interpretation
- Report

Hypothetical Case: Elements of a 10-gene NGS-based Oncology Panel

Elements Applicable	Description/Examples
Specimen Source	Solid tumor, i.e., formalin-fixed paraffin embedded (FFPE) and fresh frozen (FF) Hematological tumor, i.e., whole blood
Analyte Type	DNA RNA
Gene of Interest	5 CDx genes and 5 Non-CDx genes
Gene Variant Category	Single nucleotide variants (SNVs): 100 Insertion/Deletions (indels): 10 Fusions/Translocations: 50 Gene Amplifications: 10
Genomic Context for Gene Variant	Simple Complex (e.g., homo-polymer)
Cancer Indication	Colorectal Cancer (CRC) Non-Small Cell Lung Cancer (NSCLC)

Hypothetical Case: A 10-gene NGS-based Oncology Panel

- FDA is considering entire test system validation
  - From specimen collection, sample preparation down to the individual steps in the sequencing pipeline, and to the generation of result report
- Validation studies should be designed to demonstrate the performance characteristics of the device within the context of the intended use population

Challenges for NGS-based Oncology Panels

- What genes and associated variants should be included in the panel? How to qualify a gene/variant for inclusion?
- Limitation on reporting? Pre-defined reporting vs. de novo reporting
- Unit of validation: specimen source, analyte type, specific gene variants, specific exons, variant categories, genomic landscape?
- What is the most difficult unit(s) to validate?
- Somatic vs. germline: based on allelic frequency? Compared to matched blood?

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A Potential Intended Use for a NGS-based Oncology Panel

- The *[device name]* is a qualitative in vitro diagnostic test that uses high throughput parallel sequencing technology intended to detect sequence variations using the *[instrument name]*. The *[device name]* is indicated as an aid in characterizing sequence variations in *[xx genes]* on *[DNA and/or RNA]* isolated from *[specimen type]* specimens.
- The device is also indicated as a companion diagnostic to aid in selecting oncology patients for treatment with the targeted therapies listed in Table 1 below in accordance with the approved therapeutic product labeling.

Table 1

Gene	Variant Status	Tissue Types	Targeted Therapies

A Potential Intended Use for a NGS-based Oncology Panel

- Results other than those listed in Table 1 are only intended for patients who have already been considered for all appropriate therapies (including the ones listed in Table 1). Safe and effective use has not been established for selecting therapy using this device for the variants in the associated tissue types not listed in Table 1. Analytical performance has been established for the variants listed in Table 2 below.

Table 2

Gene	Variants	Sample Type (e.g., FFPE, fresh frozen)	Tissue Type (e.g., lung, skin)	LoD (based on LoD and reproducibility data)

- The device is not intended to be used for standalone diagnostic purposes, screening, monitoring, risk assessment, or prognosis.

Questions for Discussion Regarding the Intended Use Statement

- Does the general intended use statement above capture the necessary elements to be able to use and interpret an NGS-based oncology panel correctly?
- Should tissue types (e.g., lung, skin, etc.) be included in Table 2?
- What level of analytical validity should be established for variants reported by the assay but not included in tables 1 or 2?
- What level of clinical validity should be established for any gene reported by the assay? Would evidence of a clinical trial (NCT number) be sufficient?
- What types of warnings or disclaimers should be included for variants reported by the assay but not included in tables 1 or 2?
- What warnings or disclaimers should be included for de novo variant reporting as opposed to pre-defined variant reporting?

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Pre-Analytical and Quality Metric Approaches

- Deviations in sample preparation and processing can have large effects on the outcome of nucleic acid based test

- Traditionally, FDA has asked clinical specimens from all specimen types specified in the intended use statement to be individually validated; and critical processing parameters be assessed

- It is not clear whether information about each processing parameter across each tissue type is needed to support the claims of NGS-based oncology panels intended to be used across all tissue of origin

Pre-Analytical and Quality Metric Approaches

FDA is seeking input on whether there are suitable pre-analytical tests, representative sets of sample types, and QC-metrics that may be used instead of requiring all sample types and processing parameters to be assessed to demonstrate robustness for a particular NGS-based oncology panel.

Examples of Questions for Discussion Regarding Pre-analytical and Quality Metric Approaches

- Are there pre-analytical steps that are most critical for NGS-based oncology panel performance?

- Are there tumor types that are more challenging for NGS-based oncology panels (e.g., brain, pancreas, etc.) and in what processing contexts (e.g., fresh frozen vs. FFPE)?

- What could be the appropriate level of validation needed to support both FFPE and fresh frozen tissue claims? For instance, should performance of the NGS-based oncology panel be validated with matched clinical samples, differently prepared cell cultures (e.g., cell cultures frozen or embedded to closely mimic how clinical samples are treated), or some other way?

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Analytical Validation and Bioinformatics

- NGS-based oncology panels report on variants over a spectrum of clinical validity, from variants of uncertain significance, to variants with companion diagnostic indications linked to specific therapies
- FDA is seeking input on the appropriate level of analytical validity that should be demonstrated for variants included on NGS-based oncology panels

Examples of Questions for Discussion Regarding Analytical Validation and Bioinformatics

- Should the number of variants being reported by an NGS-based oncology panel determine whether a representative variant approach to analytical validation is acceptable? If not, are there other validation approaches that should be considered?

- Are there parameters (e.g., variant type, variant size, local sequence context, global sequence context, other) that are most important to capture in a representative variant set? Are there differences in sequencing platform that would impact selection of a representative variant set?

- Once analytical validity has been satisfactorily established for a specific set of variants, are there requirements or controls that should be in place to add, subtract, or substitute variants from the panel?

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Companion Diagnostic vs. Non-Companion Diagnostic Gene/Variant(s)

- Companion diagnostic
  - Gene/variant(s) that are intended to guide therapy with a corresponding therapeutic for a specified indication
  - The clinical claim would be stated in the intended use statement of an NGS-based oncology panel assay
  - Categorized into either traditional/first-of-a-kind or follow-on companion diagnostics
- Non-companion diagnostic
  - Gene/variant(s) that may be prognostic of clinical outcome, predictive of therapy response, or aid in the selection of therapies while not being essential for the safe and effective use of a therapeutic product
  - Understanding the status of these gene/variant(s) may aid in the management of cancer patients
  - May be reported based on sufficient analytical validation data without accompanying clinical performance data

Questions for Discussion Regarding Companion Diagnostic vs. Non-Companion Diagnostic Gene/Variant(s)

- What are key considerations for evidence that would or would not be sufficient for a follow-on companion diagnostic claim?
- What are appropriate expectations for routine reporting of gene/variants without established companion diagnostic claims?
- What are the warnings or disclaimers that should be considered around issues of panel comprehensiveness?
- What level of validation should be needed to move a variant from table 2 of the intended use to table 1 when new targeted therapeutics are approved?
- What are the warnings or disclaimers that should be considered around de novo variant reporting of unknown clinical significance?

Conclusions

- Video archive of this workshop will be posted next week
- Discussion materials are posted to our website
- Please comment on discussion materials prior to March 28, 2016
- Comments can be made to the federal docket or via e-mail

http://wcms.fda.gov/FDAgov/MedicalDevices/NewsEvents/WorkshopsConferences/ucm480046.htm

References

- FDA website on companion diagnostics: http://www.fda.gov/companiondiagnostics

- FDA companion diagnostic guidance: http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM262327.pdf

Thank You

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Panel Discussion Topic 1
Pre-Analytical and Quality Metric Approaches

- **Moderator: Aaron Schetter, Ph.D.**
- **Panelists:**
  - **John Pfeifer, M.D., Ph.D. (Washington University)**
  - **Dara Aisner, M.D., Ph.D. (University of Colorado)**
  - **Michael Berger, Ph.D. (Memorial Sloan Kettering)**
  - **Rajyalakshmi Luthra, Ph.D. (MD Anderson Cancer Center)**
  - **Michael Rossi, Ph.D. (Emory)**

John Pfeifer, M.D., Ph.D.

Washington University

Workshop: Next Generation Sequencing-Based Oncology Panels

Panel 1: Pre-analytical and Quality Metric Approaches

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Department of Pathology

Washington University School of Medicine

Disclosures

Consultant:

- Agilent; Strand Diagnostics

Co-founder:

- PierianDx; P&V Licensing, LLC

Academic affiliation:

- Washington University School of Medicine
- Genomics and Pathology Services (NGS reference lab owned by the Department of Pathology)

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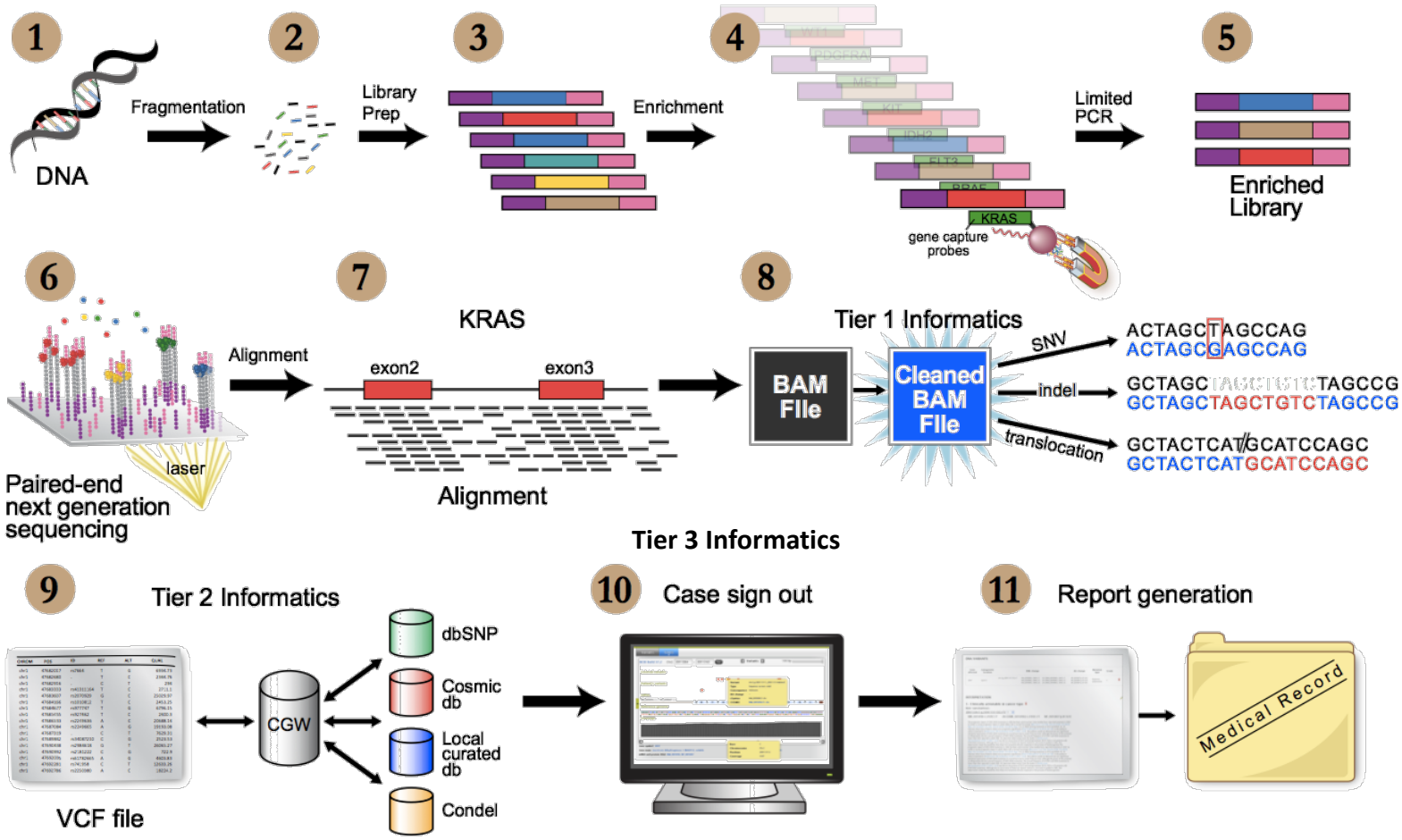
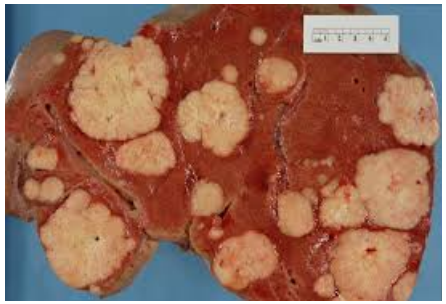
CLINICAL TESTING • RESEARCH • EDUCATION

Overview of clinical NGS



Reference: Cottrell CE, et al. *J Mol Diagn* 2014;16:89-105

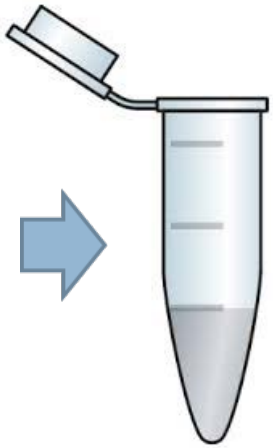
There are numerous pre-analytical and quality concerns before step #1…



Gross processing

Tissue processing

Histopathologic review and tumor enrichment

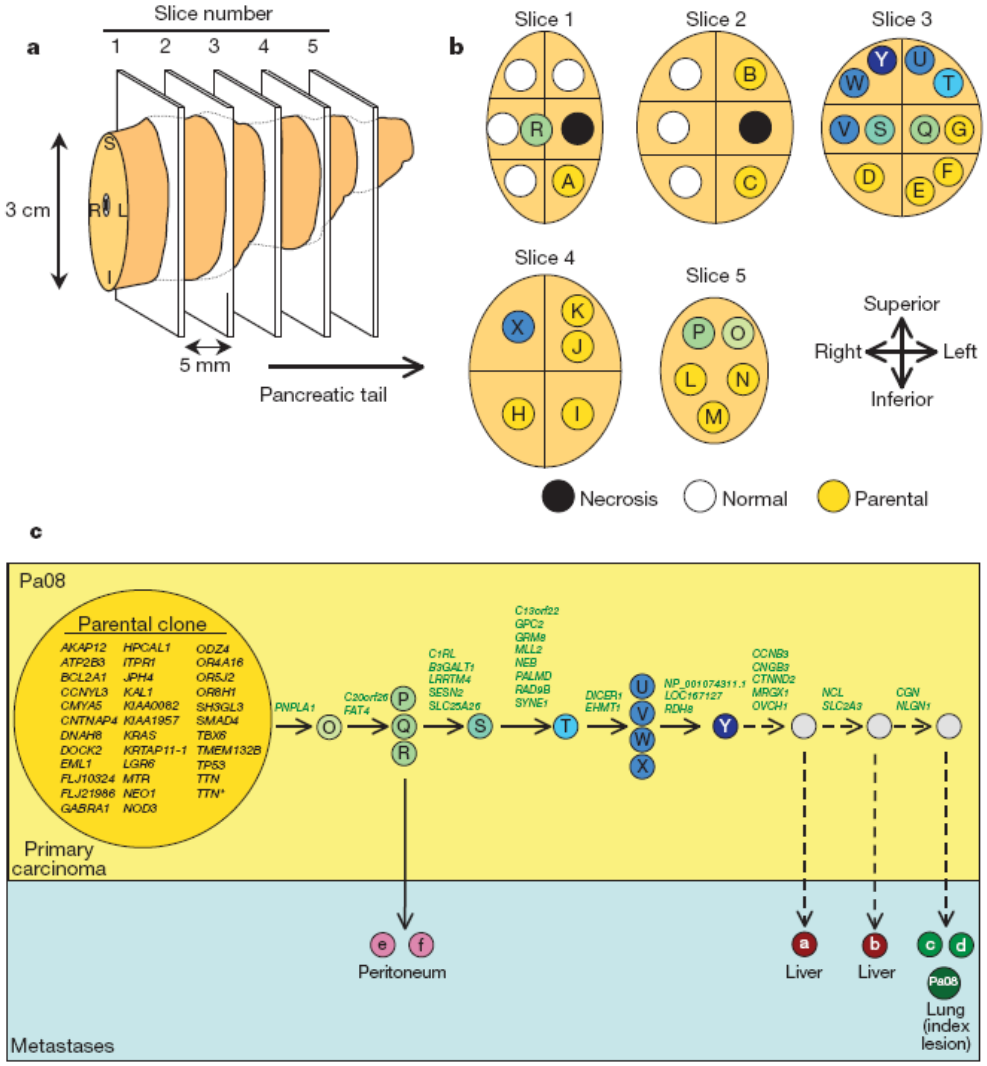


DNA extraction

… even before library preparation, bioinformatic pipeline, variant interpretation, and reporting.

Tumor sample: site

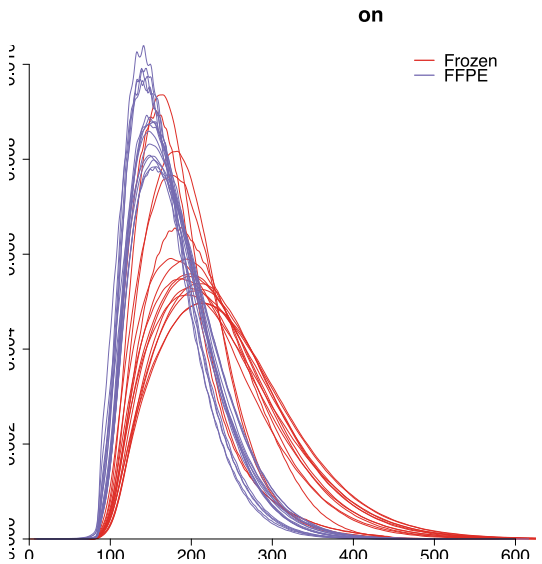
- Where to sample the primary tumor? How many times?
- Sample the metastasis instead? Which metastasis? How many times?
- Need for paired tumor-normal tissue samples? What is the "normal"?

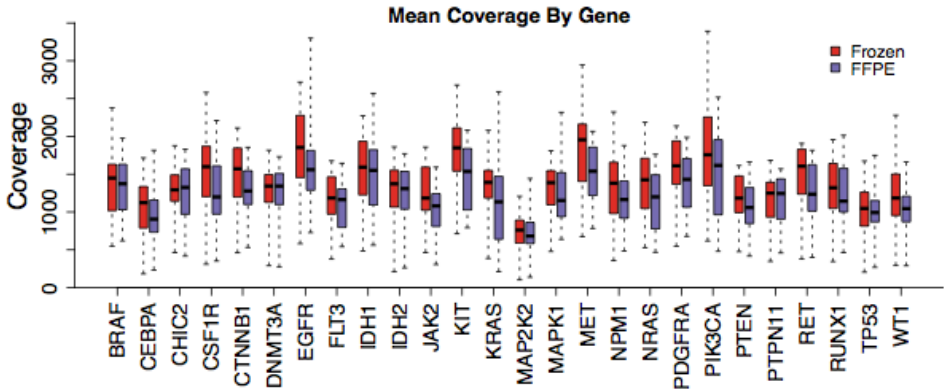


Reference: Yachida S, et al. *Nature* 2010; 467:1114-1117

NGS works from clinical FFPE tissue

- Compared 17 paired fresh frozen and FFPE lung adenocarcinomas
- Oncology gene panel (hybrid capture based)
- Sequenced on HiSeq to an average depth >1,000x
- Difference in mean insert size
- No difference in coverage



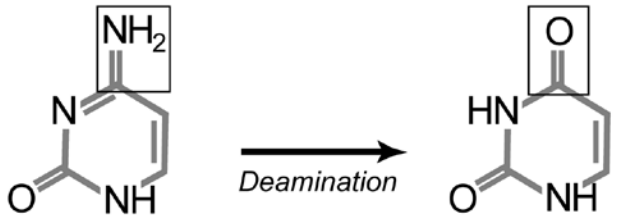


Reference: Spencer D, et al. *J Mol Diagn* 2013;15:623-633

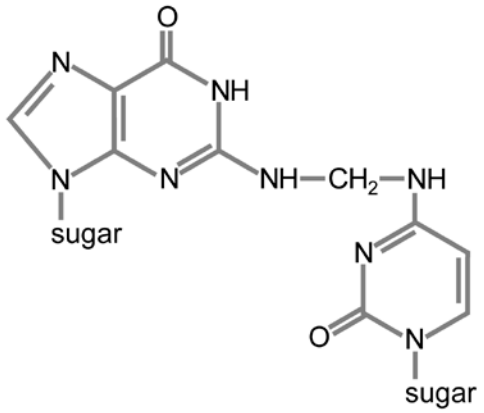
FFPE tissue has chemical artifacts

- FFPE causes a number of chemical changes in DNA (and RNA) including deamination, oxidation, cyclic base derivative formation and methylene crosslink formation

- In FFPE tissue, transitions (including at CpG dinucleotides) have a rate of 0.0015 and are about 4X more common than in fresh tissue



Cytosine **Uracil**

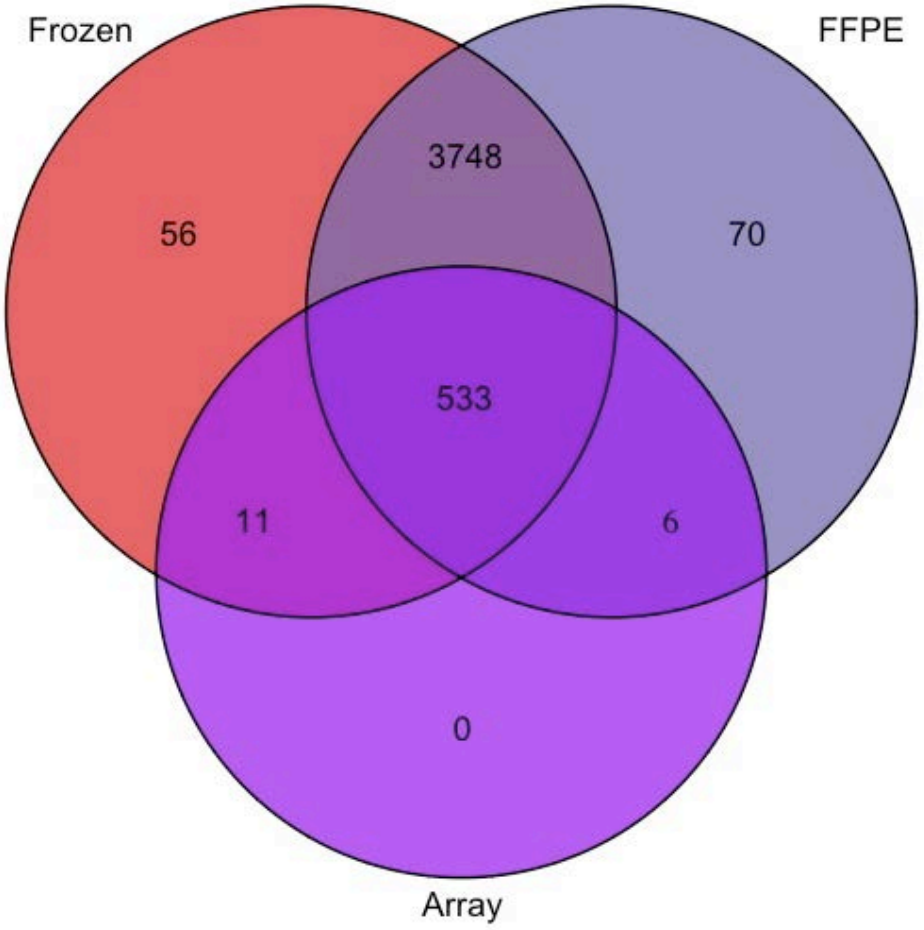


Guanine **Cytosine**

Reference: Spencer D, et al. *J Mol Diagn* 2013;15:623-633

NGS of paired FFPE and fresh tissue has high concordance

- Concordance between FFPE and frozen tissue >99.99% for all positions
- 98.6% concordance for SNVs calls between FFPE and frozen tissue
- 100% Concordance between Array and NGS
- Thus, variants unique to FFPE/frozen tissue likely represent tumor heterogeneity



Reference: Spencer D, et al. *J Mol Diagn* 2013;15:623-633

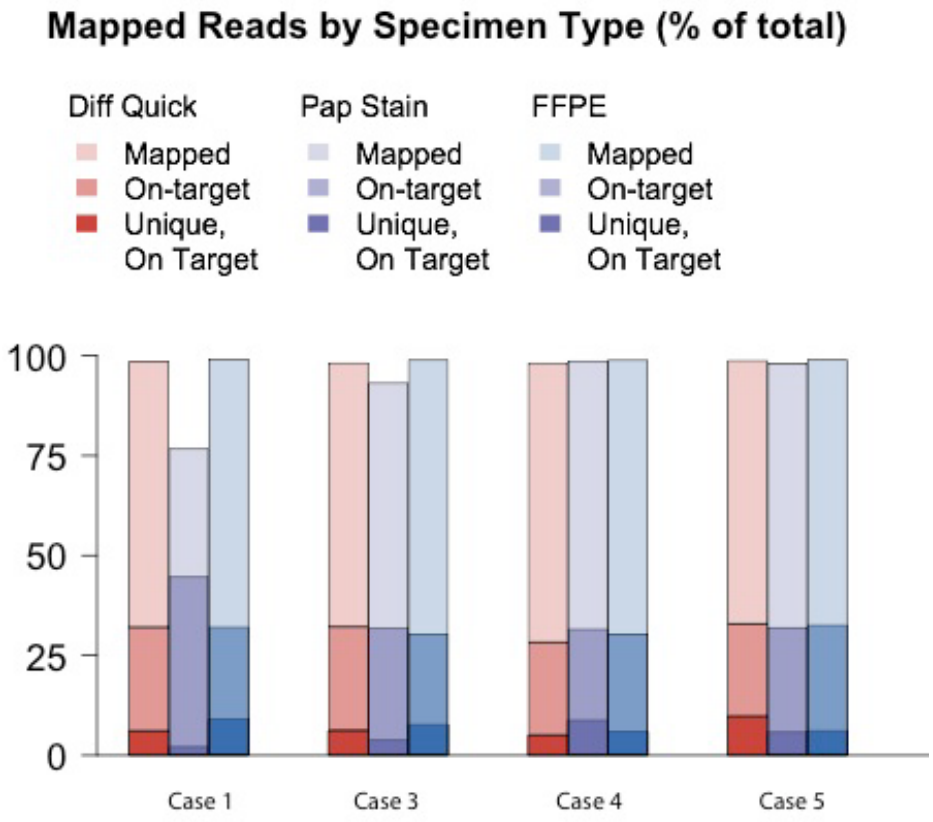
Prolonged ischemic time and prolonged formalin fixation don't make a major difference in overall sequencing results

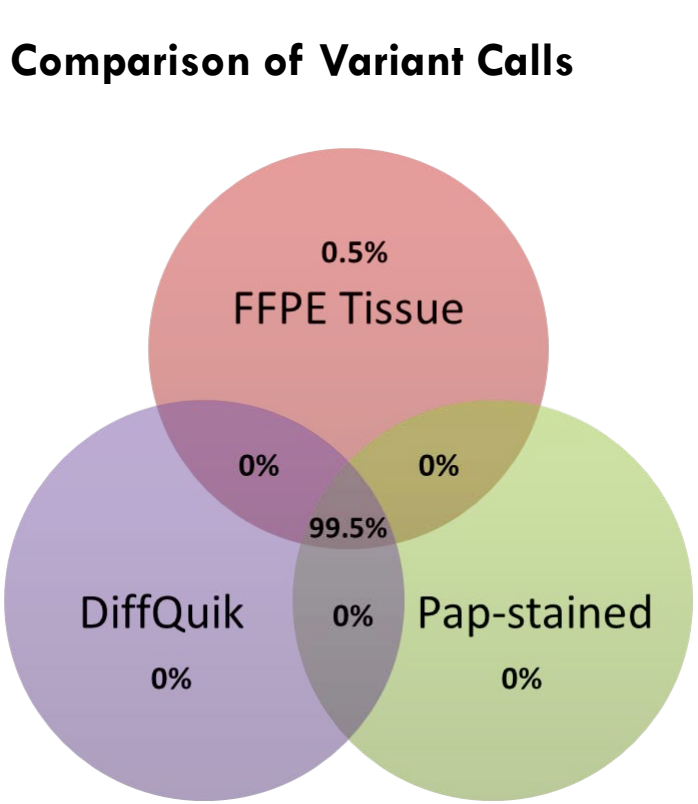
- No major difference in percentage of mapped reads
- No major difference in on-target reads

- Increased coverage variability
- Lower number of unique reads

Reference: Spencer D, et al. *J Mol Diagn* 2013;15:623-633

NGS also works from cytology samples

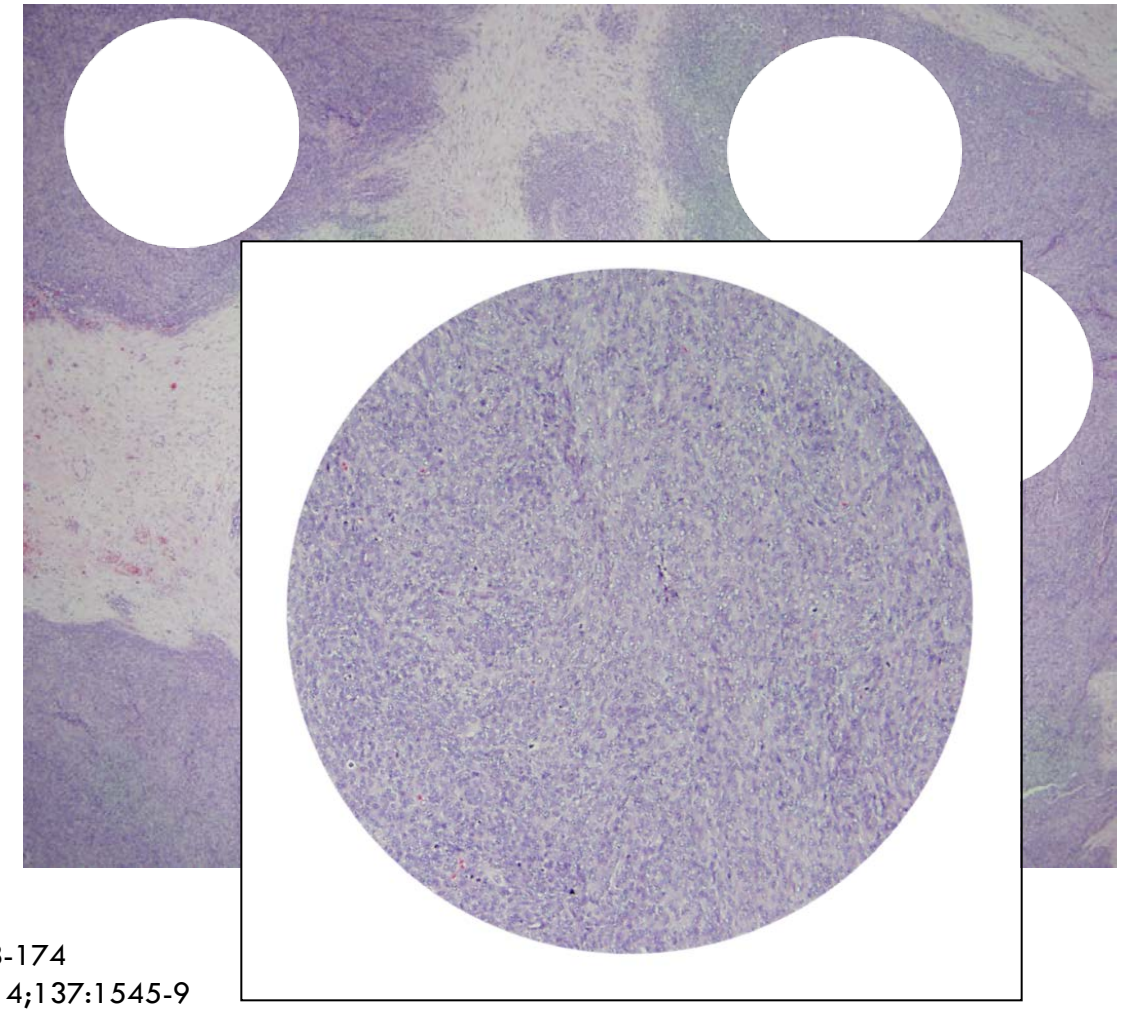




Reference: Karnes H, et al. *Cancer Cytopathol* 2014;122:104-113

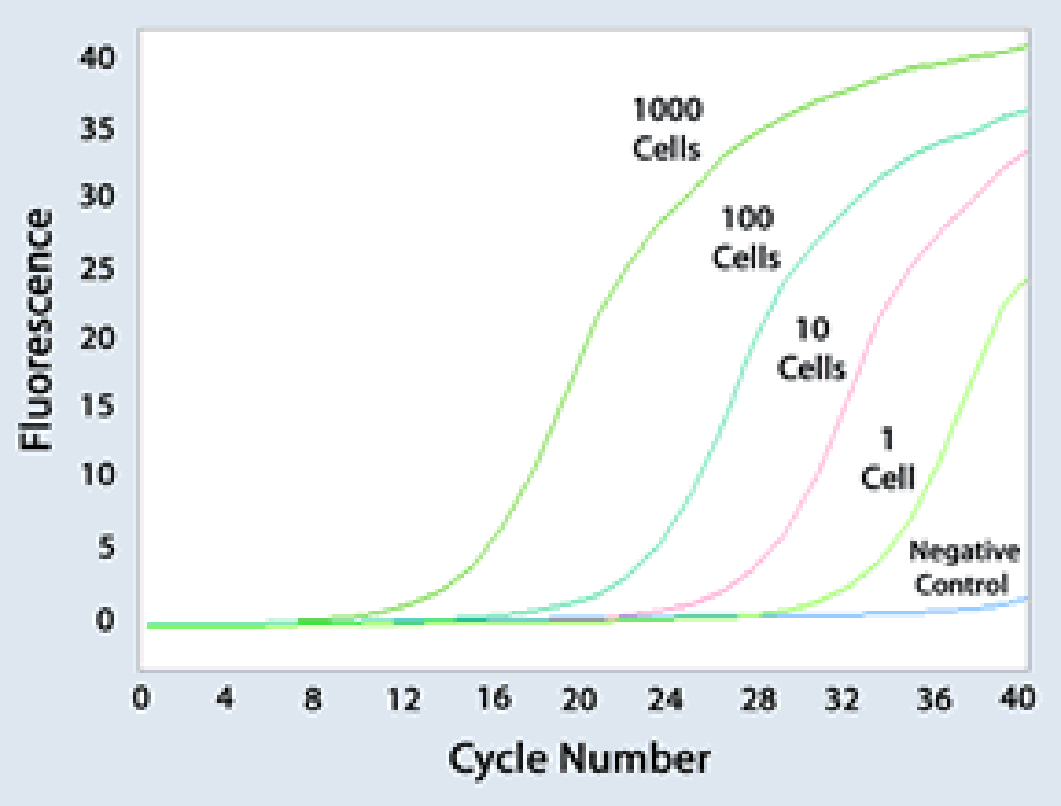
Tumor sample: cellularity, viability, and enrichment

- Need for histopathologic review to identify regions of tumor, assess tumor cellularity, and assess tumor viability
- Is a significant source of variability
- Microdissection can achieve high tumor cellularity (can be via a tissue scroll, manual microdissection, or needle core)

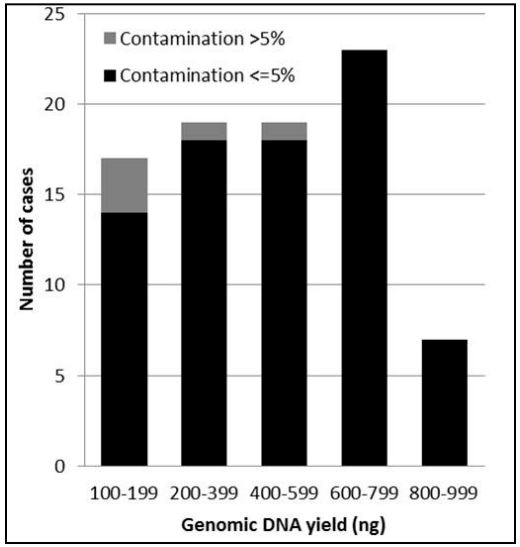


References: Smits AJ, et al. *Mod Pathol* 2014;27:168-174
Viray H, et al. *Arch Pathol Lab Med* 2014;137:1545-9

Tumor sample: DNA requirement

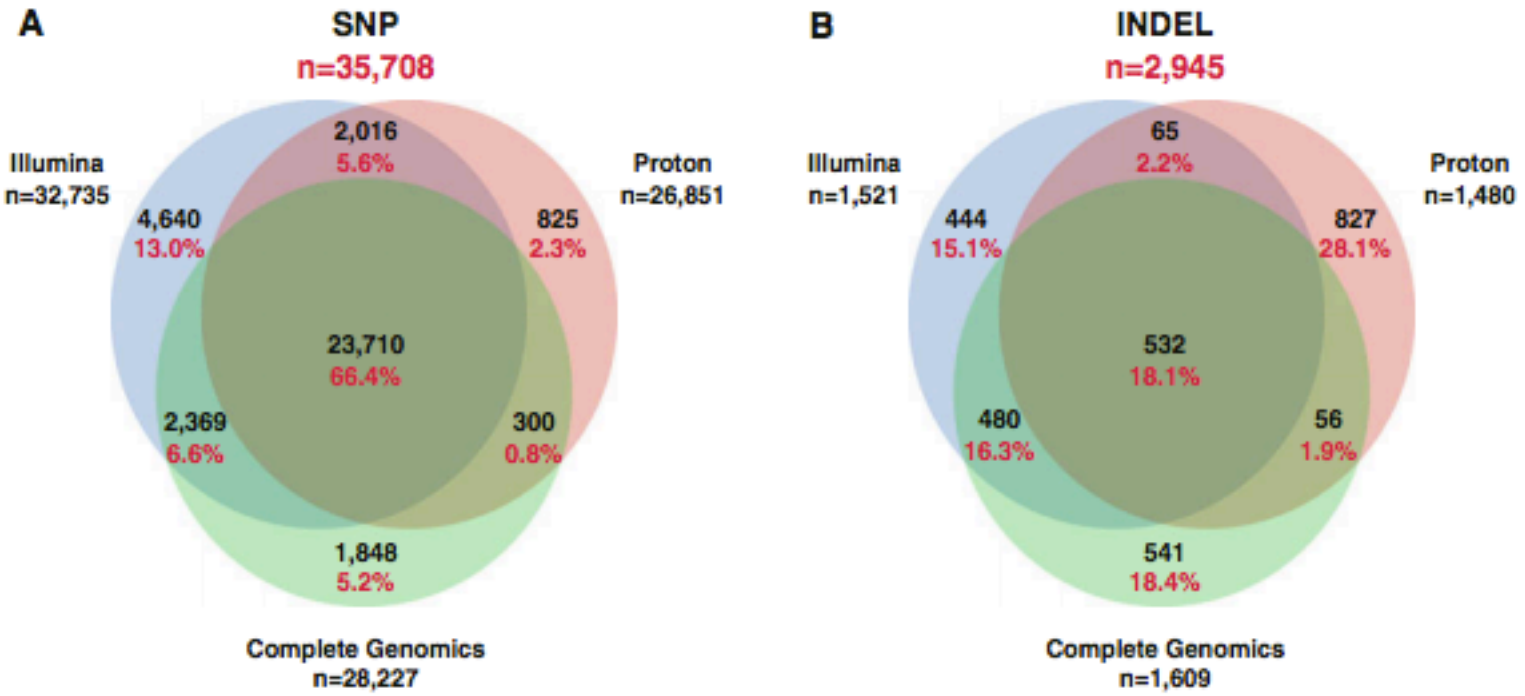


- Need to achieve necessary quantity of DNA (in our experience, 6% of FFPE cases have <10ng of DNA, 13% <100ng, 25% <200ng, and 57% <750ng)
- Library complexity is not the same as depth of sequence
- Emerging issue of specimen provenance



Reference:s Al-Kateb H, et al. *Mol Onc* 2015;9:1737-1743
Sehn JK, et al. *Am J Clin Pathol* 2015;144:667-674
Mathias P, et al. *Am J Clin Pathol* In press

Different platforms yield different results



Reference: Boland JF, et al. *Hum Genet* 2013;132:1153-1163

Sample types for validation studies…

Patient samples

- limited supply
- not comprehensive

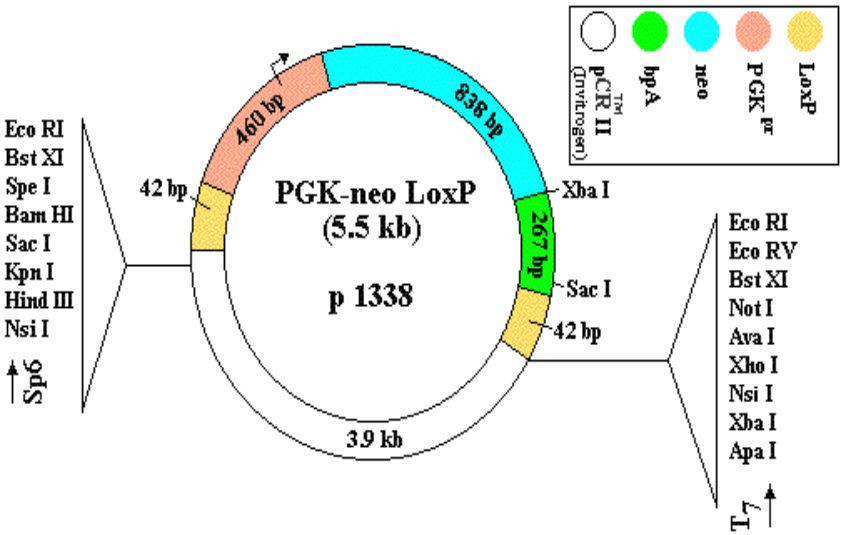
Cell lines

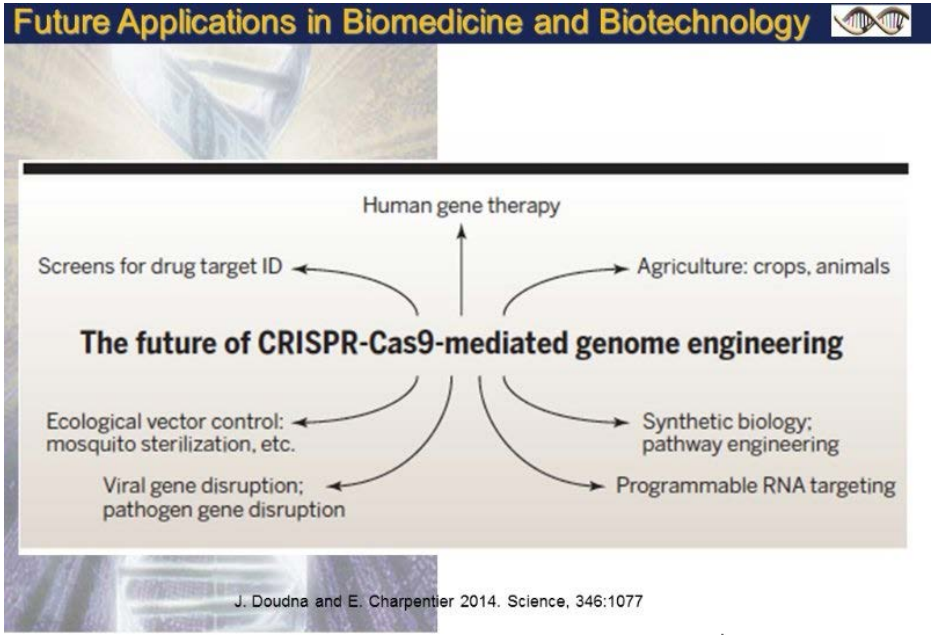
- complex mixtures possible
- wide variety of variants, but not comprehensive
- inexhaustible
- many commercial and NFP sources

…include engineered constructs and cell lines

Engineered constructs and cell lines

- complex mixtures possible
- wide variety of variants, but still not comprehensive
- expensive to produce, but many commercial sources
- inexhaustible
- hard to model different VAFs
- sequence artifacts

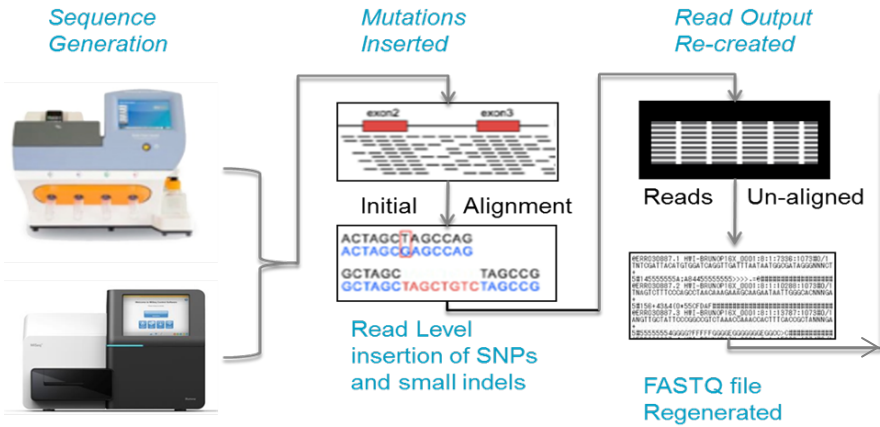




“in silico” datasets

Sequence files that have undergone in silico mutagenesis (i.e., sequence files from NGS of a well characterized specimen that have been manipulated by computerized algorithms to introduce relevant sequence variants into the reference sequence files) have advantages:

- mixtures of variants and VAFs characteristic of inherited diseases and cancer
- inexpensive, comprehensive, current
- challenge an NGS test's bioinformatic pipeline from alignment through variant detection, annotation, and interpretation (but therefore supplement but do not replace traditional methods)
- broad applicability



References: Frampton M et al. *PLoS One* 2012;7: e49110
Schrijver I et al. *J Mol Diagn* 2014;16:283-287
Kalman LV et al. *Arch Pathol Lab Med* 2013;137:983-988
Duncavage et al. *J Mol Diagn* 2015;17:797

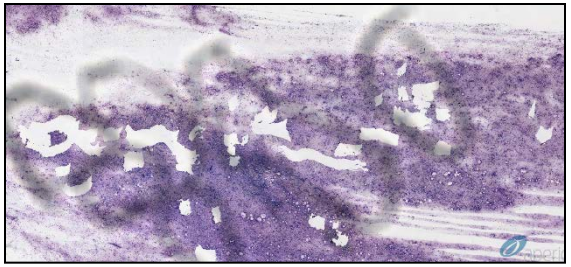
Dara Aisner, M.D., Ph.D.

University of Colorado

Confronting and Mitigating Pre-Analytic Variability

From Specimen To NGS Data: Many Areas of Pre-Analytic Variability

Step	Sources of Variability
Tissue Acquiring Procedure	• Immediate transfer medium • Temperature • Cold ischemic time • ? Target organ • ? Procurement method
Origin Tissue	• Matrix effect
Tissue receipt in processing laboratory	• Fixative solution • Cytopathology specimens – spectrum of handling approaches
Processing	• Heat/pressure • Microwave • Smear • Cytospin • Other • Fixative time, other reagents



From Specimen To NGS Data: Many Areas of Pre-Analytic Variability

Step	Sources of Variability
Specimen Assessment	• Subjective assessment of tumor cellularity • Subjective assessment of best approach for tumor enrichment • Subjective assessment of total quantity to utilize
Tumor enrichment	• Coring/macroscopic isolation directly from block • Microdissection using guide slides • Microscopic microdissection • Slide scrape (cytology) • Other
Extraction	• DNA only • RNA only • TNA • Other • Many methods/commercial options for each
Assessment of extract quality	• qPCR for fragmentation • Microgel fragmentation assessment
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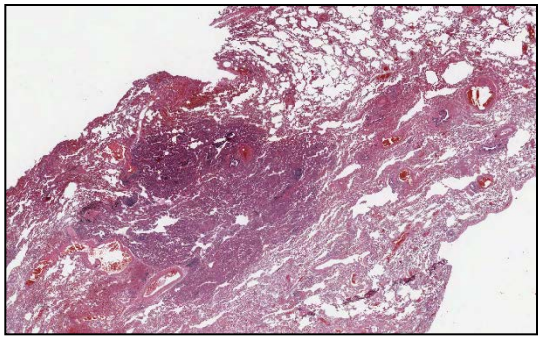
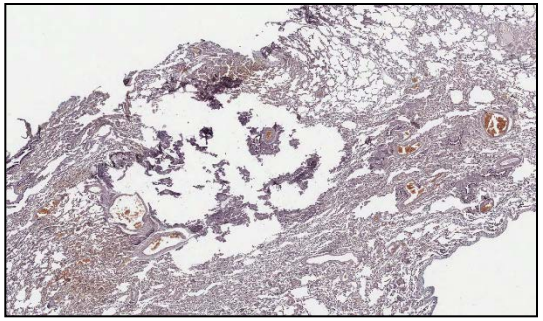
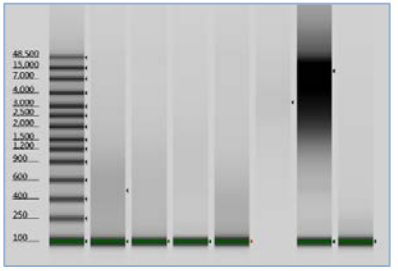




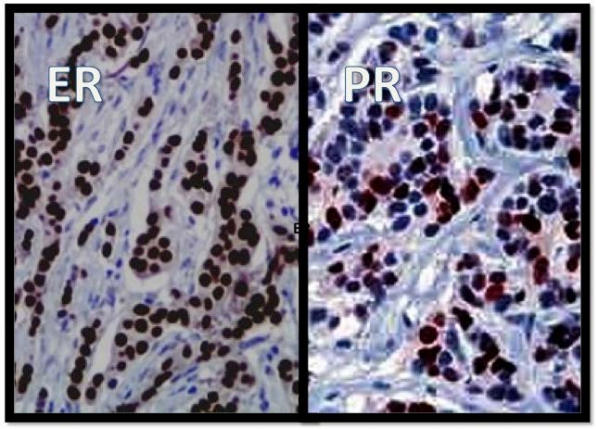


Confronting Variability

Step	Sources of Variability
Tissue Acquiring Procedure	• Immediate transfer medium • Temperature • Cold ischemic time • ? Target organ • ? Procurement method

These are not variables that FDA is likely to be able to stipulate in a working medical environment

- Professional guidelines have become effective
  Example: stipulating cold ischemic time for samples of breast cancer for subsequent ER, PR and *ERBB2* (*HER2*) analysis



Breast-cancer.ca

Mitigating factor:

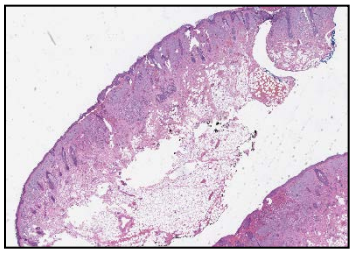
- These sources of variability are less likely to impact DNA - based testing (compared to other analytes)

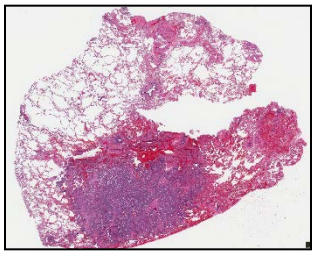
Solution:

- Identify quality metrics that can be applied after this stage for specimen evaluation

Confronting Variability

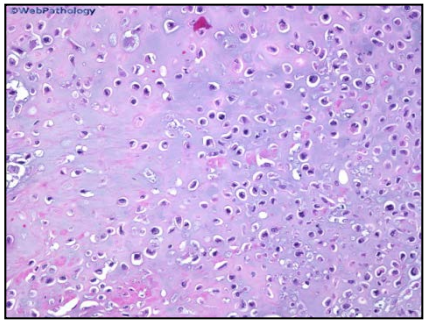
Step	Sources of Variability
Origin Tissue	• Matrix effect



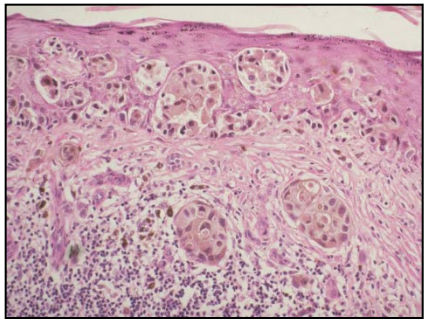


- To what degree is it necessary to validate tissue origins separately?

- Are colon, lung, skin, liver etc. sufficiently different to suggest that each organ type needs a separate validation?



- Most tissues are highly equivalent in **core constituents**: epithelium/parenchyma, inflammatory cells, red blood cells, stromal cells



- Probably not necessary to think about different organs as much as different matrices with potential interfering impact

- Is there a matrix effect of: Mucin? Chondroid? Melanin? Others?

- Validation should focus less on distributing across tissue types and more on matrix effects **outside of core tissue constituents**

Confronting Variability

- The extent of variability here is substantial
- Over-prescribing these variables will lead to lack of access to testing for substantial proportions of patients and will interfere with the medical practice of pathology (these tissues are also used for diagnosis)

Step	Sources of Variability
Tissue receipt in processing laboratory	• Fixative solution • Cytopathology specimens – spectrum of handling approaches
Processing	• Heat/pressure • Microwave • Smear • Cytospin • Other • Fixative time, other reagents

- Mitigating factor: There are mechanisms to evaluate nucleic acid integrity
- **Solution: Establish metrics that look at the resulting product (nucleic acid extract and/or NGS data)**

Confronting Variability

Step	Sources of Variability
Specimen Assessment	• Subjective assessment of tumor cellularity • Subjective assessment of best approach for tumor enrichment • Subjective assessment of total quantity to utilize

This is fundamentally the practice of laboratory medicine

- Professionals who evaluate tissue for a living are MUCH more skilled at determining how to get the right tissue into the tube (compared to generic instructions)

From an FDA approved package insert

Sample requirements

- Standard formalin-fixation and paraffin-embedding procedures should be followed. To limit the extent of DNA fragmentation:
  - Fix tissue samples in 10% formalin as quickly as possible after surgical removal.
  - Use a fixation time of 14–24 hours (longer fixation times lead to more severe DNA fragmentation, resulting in poor performance in THxID™-BRAF assay).
  - Thoroughly dehydrate samples prior to embedding (residual formalin can inhibit the Proteinase K digestion).
- Sections will be processed according to the pathologist's indications:
  1. If the sample section contains **more than 80%** of tumor cells **and** does not contain a distinct area of necrotic tissue, fatty tissue, hemorrhagic tissue or non-tumoral melanin-rich area, then the entire section can be placed in a tube, or if the sample is on a slide, it can be entirely scraped with a scalpel.
  2. If the sample section contains **less than 80%** of tumor cells, then the section must be manually macro-dissected in order to reach a final content of at least 80% tumor cells. Use a dedicated sterile scalpel to select the tissue part in order to enrich the sample in tumoral cells.
  3. If the sample section contains necrotic tissue, fatty tissue, hemorrhagic tissue or non-tumoral melanin-rich area, then the section should be manually macro-dissected. Use a dedicated sterile scalpel to select the tissue part in order to avoid the undesirable portion.
- The minimum surface of tissue required for a 10 µm section is 20 mm^2, not counting the necrotic / fatty / hemorrhagic / non-tumoral melanin-rich area if it is deemed dissectible (see above). If 5 µm sections are prepared, the minimum is then 40 mm^2. Therefore a sufficient number of sections should be included to meet this requirement, while not exceeding 8 x 10 µm sections (or 16 x 5 µm sections) to stay within the recommended limit of the purification column.
- The total surface of tissue should not exceed 250 mm^2 if 10 µm sections are prepared or 500 mm^2 if 5 µm sections are prepared.

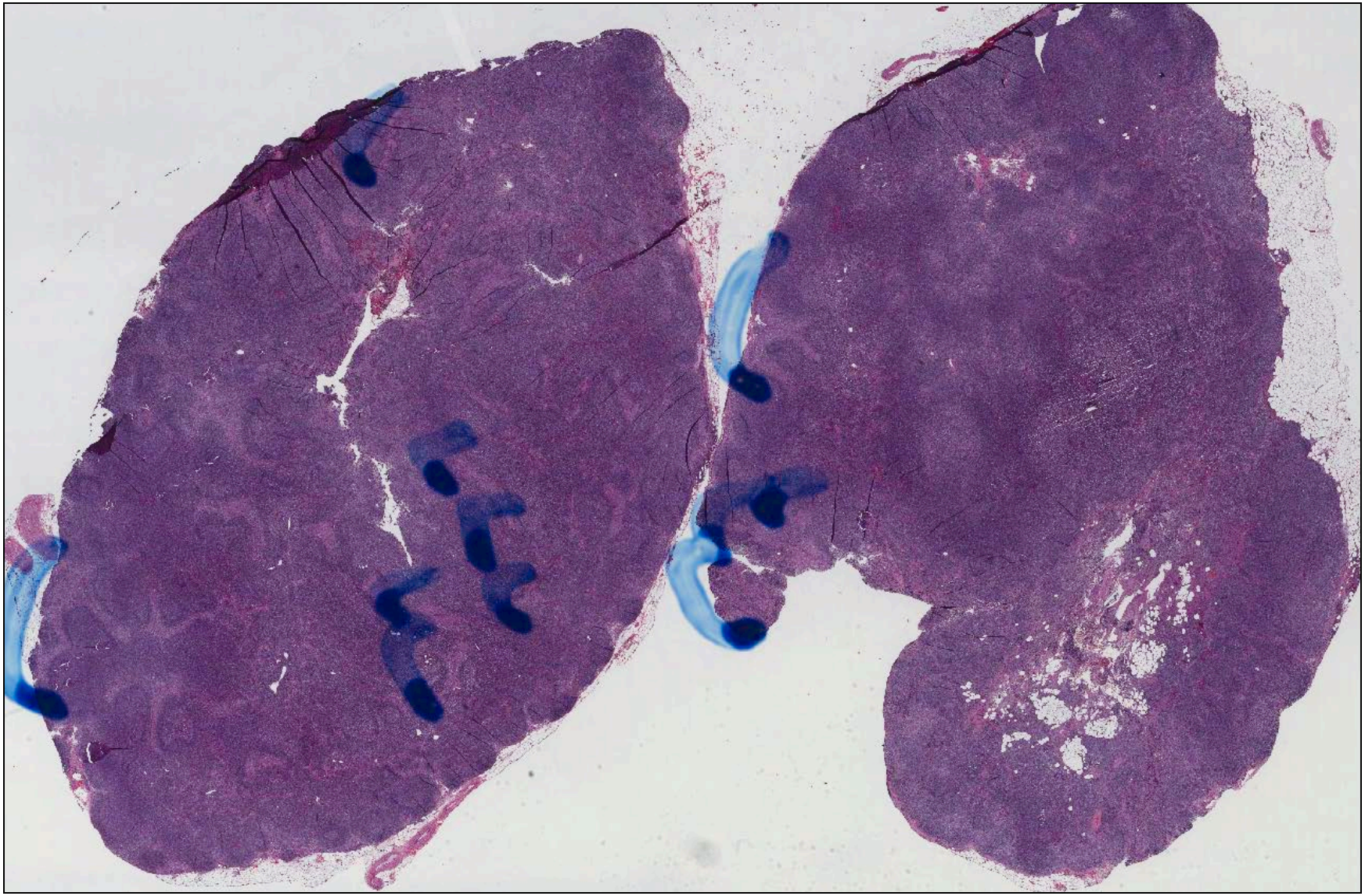
Note: Use a single scalpel per sample.

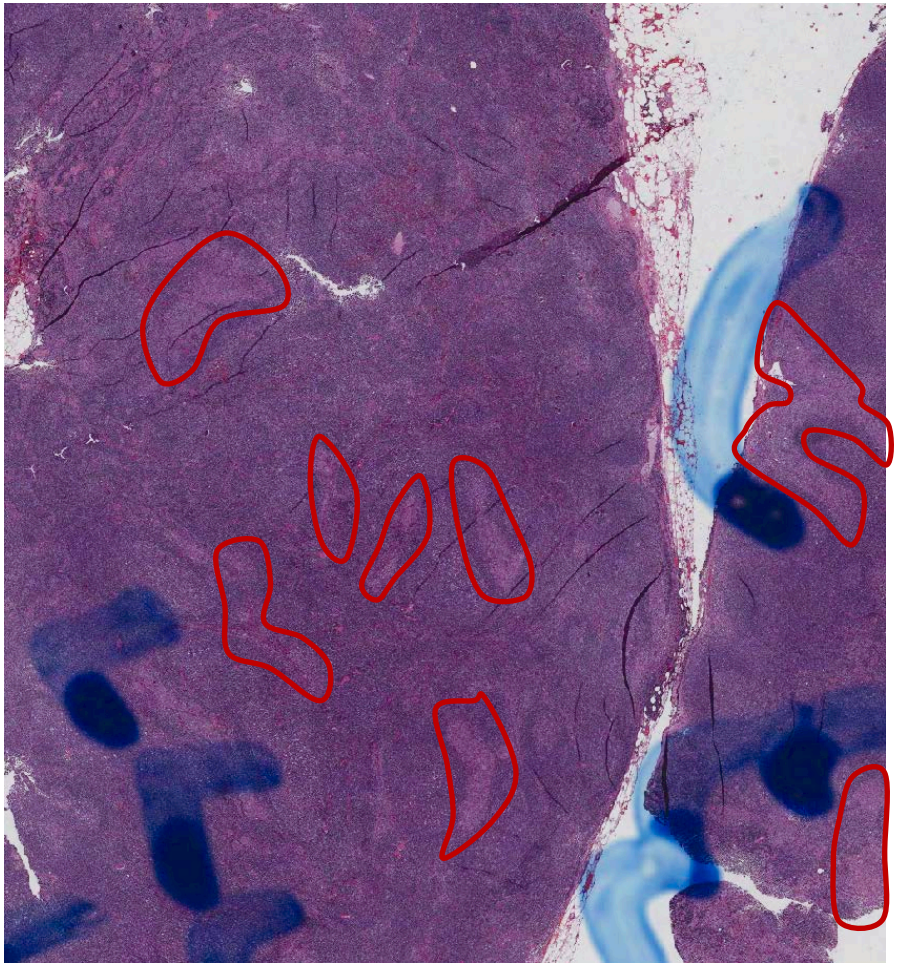
Confronting Variability

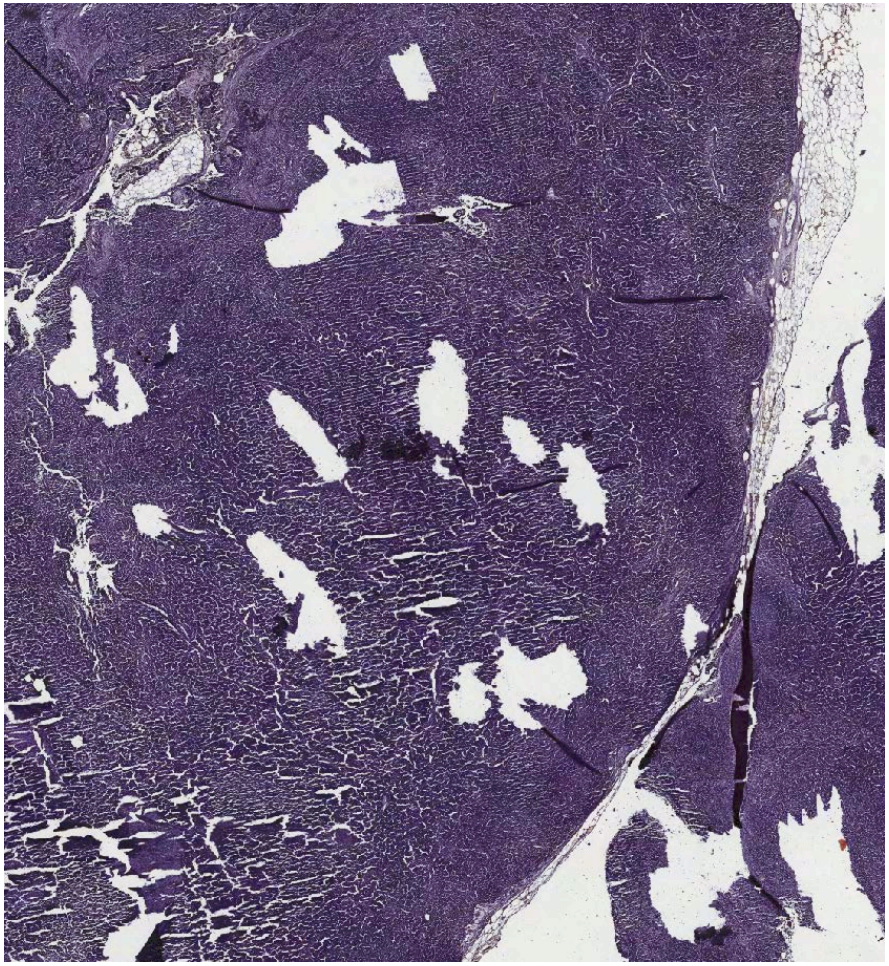
Step	Sources of Variability
Tumor enrichment	• Coring/macroscopic isolation directly from block • Microdissection using guide slides • Microscopic microdissection • Slide scrape (cytology) • Other

This is *also* the practice of medicine

- Combining the visual assessment of a tissue with the means to enrich it is a medical judgement, made for the patient, much like a radiologist makes a medical decision for a patient based on what he/she sees
- This should not be over-prescribed
- You cannot assume that samples can always be run without tumor enrichment or with only macro-dissection







Microdissection employed (12 consecutive 10 micron sections microdissected)
This specimen would have had a high probability of false negative without *microdissection*;
macrodissection would likely have insufficient tumor enrichment
BRAF c.1799T>A (p.V600E) mutation identified

Mitigating Variability

Laboratories are unlikely to migrate to a single assay platform for everything

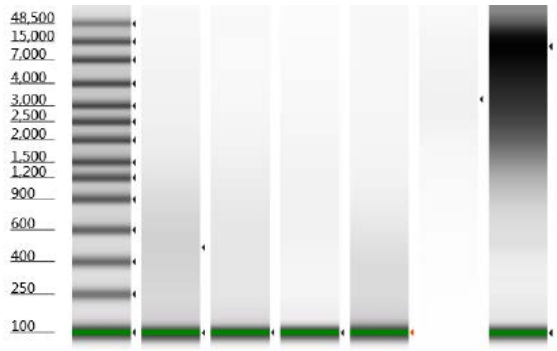
Step	Sources of Variability
Extraction	• DNA only • RNA only • TNA • Other • Many methods/commercial options for each

- Flexibility with regard to extraction approach is sorely needed in order for laboratories to have a unified approach to specimen pre-analytic processing
- Inflexible approaches will lead to:
  - Rapid tissue depletion
  - Inability to perform orthogonal assays
  - Inability to perform back up assays
  - Restrictions on new assay development
- Mitigating factors: There are methods to evaluate nucleic acid integrity

- **Solution: Establish metrics that look at the resulting product (nucleic acid extract and/or NGS data)**

Mitigating Variability

Step	Sources of Variability
Assessment of extract quality	• qPCR for fragmentation • Microgel fragmentation assessment
Input determination	• Modulated input based on extract assessment • Fixed input



- In many cases, with limited tissue, you have **ONE** chance to get this right
- Proper nucleic acid assessment is the integration of multiple pieces of data:
  - Fluorometric quantification (not spectrophotometric)
  - Microgel analysis (e.g. Bioanalyzer)
  - Real-time PCR
- There IS a subjective component here, particularly when it comes to the 'exceptions'
- None of the established methods is foolproof

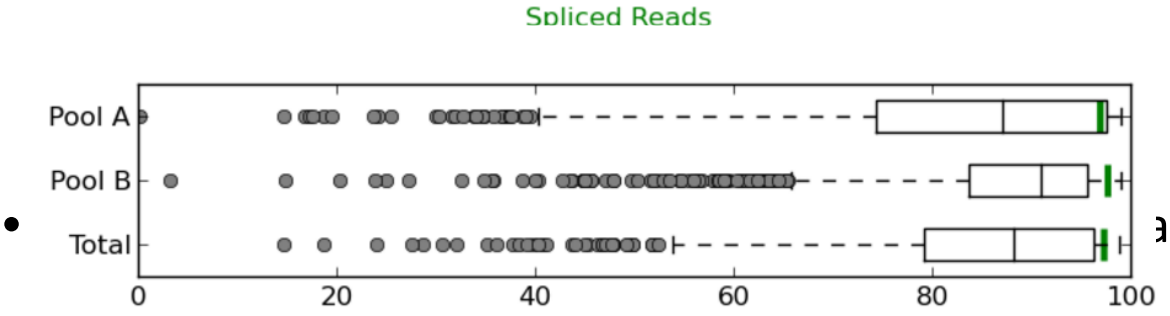
  Examples:
  - cases that 'never should' have worked but did
  - cases that clearly 'should' have worked but didn't
- The experienced practitioner can use these metrics as *guidance* (not gospel) for challenging cases

Mitigating Variability: A Key Part of Laboratory Medicine

- There are many instances when a specimen does not fit into pre-established criteria
- We owe it to the patient to take a ‘try anyway’ approach
  - Eliminating the ability to handle exceptions will eliminate access for many patients
- How do you ensure that results in the ‘try anyway’ approach are accurate?
  - The key here is the *ability to review the primary data*
  - Having practitioners ‘black boxed’ to the actual data eliminates our ability to confront and mitigate variability

How Do We Use Data to Mitigate Variability?

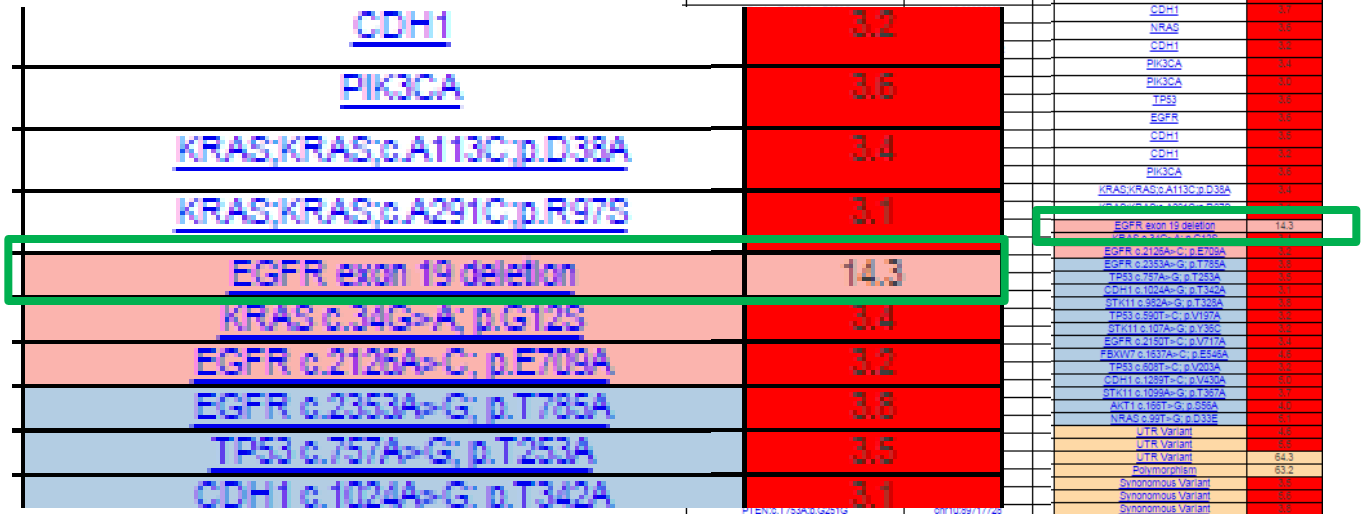
- In the setting of somatic condition + FFPE, Ti/Tv ratio is not meaningful
- Some hard metrics can be established



- ations = practice of

Example case

- 57 year old female with adenocarcinoma of lung
- Scant sample
- FFPE QC – moderate to poor quality
- NGS results show extremely high level of artifact



- Multiple places where this assay could have been 'cut off' based on *imposed* metrics
- The ability to test real patients in real situations relies on us using training, expertise and judgement

Summary

- The extent of pre-analytic variability is extremely high, and cannot be mitigated through regulatory mandate – many of these decisions are medical decisions
  - Attempts to mandate pre-analytic specimen handling will result in a high level of patients with tissue deemed unsuitable for testing
  - Need to incorporate flexibility to attempt testing, even if criteria are not met

Panel Discussion Topic 1
Pre-Analytical and Quality Metric Approaches

- **Moderator: Aaron Schetter, Ph.D.**
- **Panelists:**
  - **John Pfeifer, M.D., Ph.D. (Washington University)**
  - **Dara Aisner, M.D., Ph.D. (University of Colorado)**
  - **Michael Berger, Ph.D. (Memorial Sloan Kettering)**
  - **Rajyalakshmi Luthra, Ph.D. (MD Anderson Cancer Center)**
  - **Michael Rossi, Ph.D. (Emory)**

Quality Control Metrics

FDA is seeking panel input on the critical quality metrics that should be evaluated for NGS oncopanels

- What pre-analytical quality metrics should be used to evaluate if nucleic acids (both RNA and DNA) are suitable for NGS assays prior to library construction (e.g., nucleic acid concentration, nucleic acid purity, and/or integrity of nucleic acid)?
- For pre-analytical validation, what steps of the NGS workflow do you think should be evaluated?
- What quality control metrics from the sequencing run are most important to be evaluated and how do you use these metrics to ensure that the assay performed adequately and provide reliable results? Can these be metrics be generalized to different manufacturers?
- Under what circumstances do you think that pre-analytical validation would not require evaluation of variant calling accuracy?

Sample Processing

FDA is seeking panel input on the essential pre-analytical variables that should be tested

- What are the specific concerns that you think should be addressed when evaluating how variation in sample processing FFPE specimens may affect the output of the NGS oncopanel?
- What level of validation do you think is needed to support FFPE, fresh frozen and cytology specimen claims?
- How should differences in tumor cellularity (derived from macro-dissection or micro-dissection) be accounted for in pre-analytical quality control parameters?
- What evidence would be required to demonstrate that any nucleic acid isolation method can be used?

Pan-Cancer Claims

FDA is seeking panel input on the types of studies needed for manufactures to claim that their assay can be used across multiple cancer types.

- What representative tumor types do you recommend be tested to justify a Pan-Cancer claim?
- What tumor types have been the most difficult to get reliable NGS data from? Are there tumor types that you think should be excluded from pan-cancer claims unless the manufacturer can demonstrate that the assay has adequate performance in that tissue type?
- What level of validation should be needed to add or modify specimen types (tissue source and/or tissue sample processing) for an already approved NGS-based oncology panel?

Sample Types for Analytical Validation Studies

FDA is seeking panel input on how contrived samples may be used to demonstrate the analytical validity of an NGS-based oncology panel.

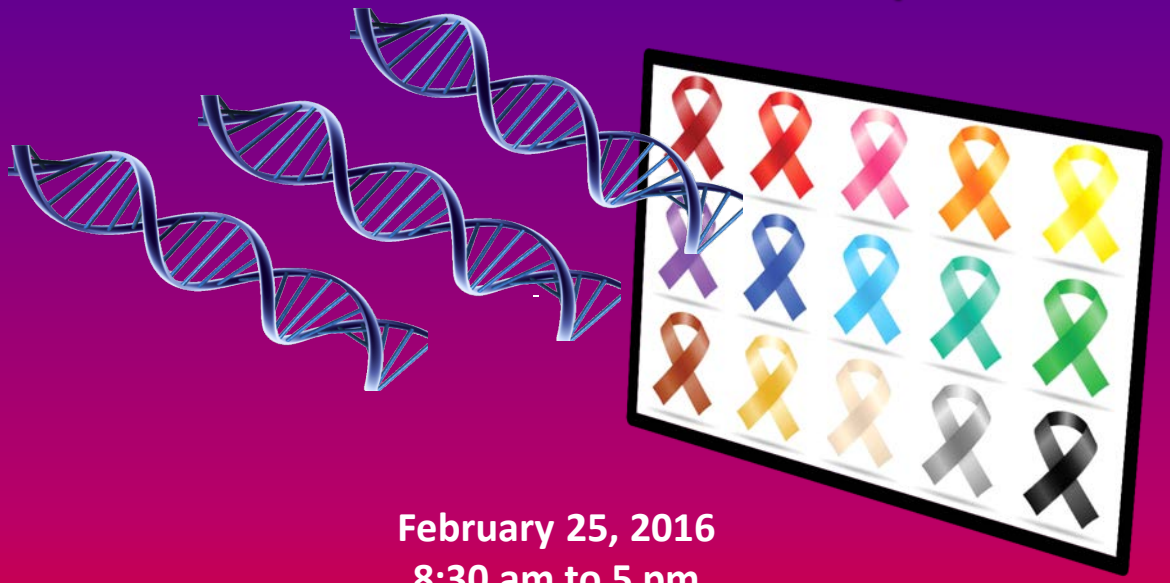
- What types of commutability studies should be conducted in order to infer the performance of the assay on clinical samples from data obtained in cell lines or plasmids?
  - What quality metric similarities and differences would you expect to see?
  - Would you expect to make calls with more confidence in contrived samples and how could studies be adjusted to more closely mimic clinical scenarios?
- When clinical samples and cell lines with specific variant types are not attainable, and with the understanding that plasmids lack the 3D architecture of genomic DNA, should engineered cell lines be the preferred method of contriving samples for analytical validation purposes?

BREAK

10:30-11:00 am

FOOD AND DRUG ADMINISTRATION

Next Generation Sequencing-based Oncology Panels Public Workshop



February 25, 2016
8:30 am to 5 pm

White Oak, MD

Webcast address: https://collaboration.fda.gov/ngsop0216/

FDA's Medical Devices News & Events Workshops & Conferences calendar:
http://www.fda.gov/MedicalDevices/NewsEvents/WorkshopsConferences/default.htm

Panel Discussion Topic 2
Analytical Validation and Bioinformatics

Moderator: Donna Roscoe, Ph.D.

Panelists:

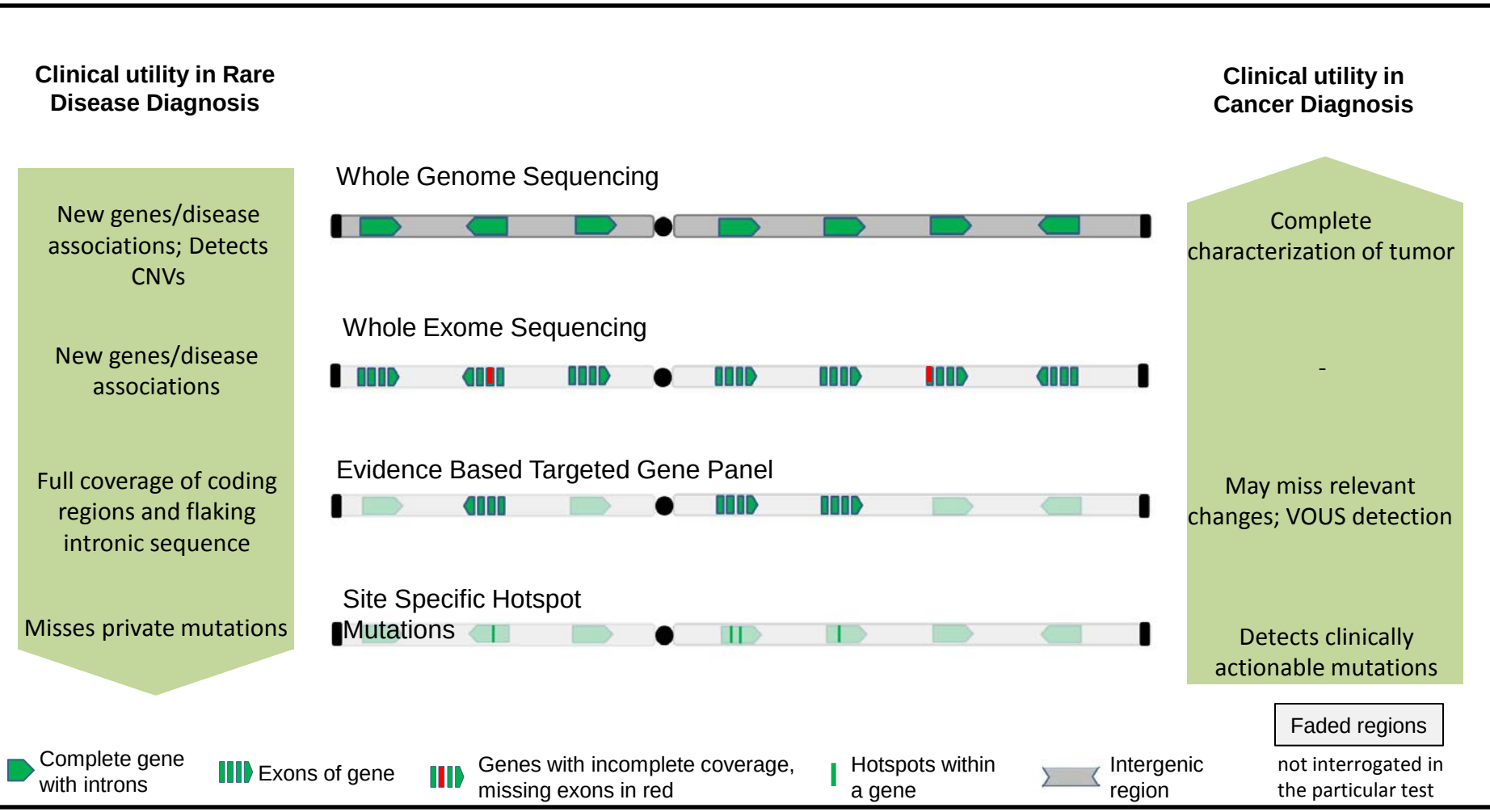
- **Madhuri Hegde, Ph.D. (Emory)**
- **Eliezer M. Van Allen, M.D. (Dana-Farber Cancer Institute)**
- **Josh Deignan, Ph.D. (UCLA)**
- **David Eberhard, M.D., Ph.D. (UNC, Chapel Hill)**
- **Robert Klees, Ph.D.(New York State Dept. of Health)**

Madhuri Hegde, Ph.D.

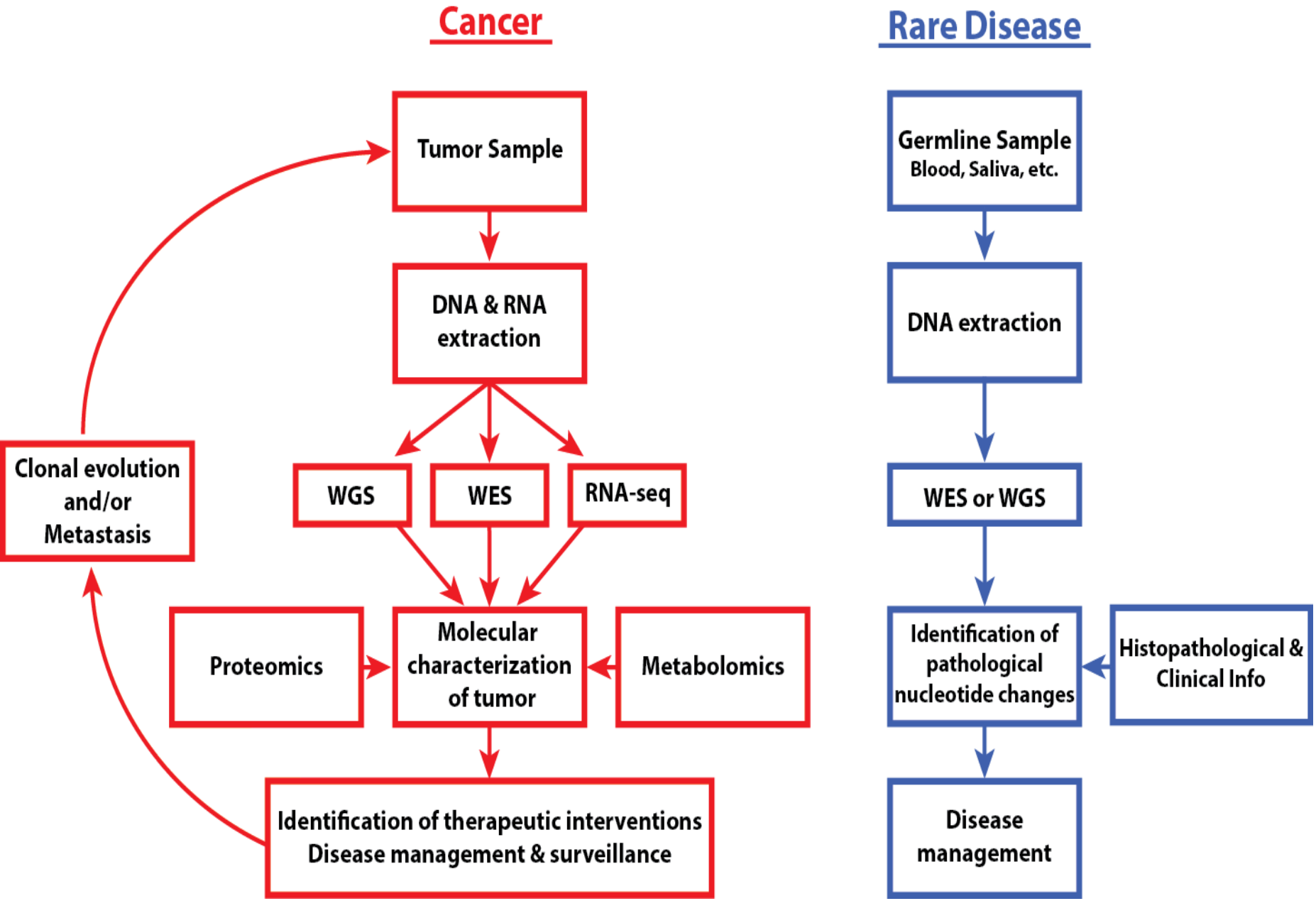
Emory University

Analytical Challenges in NGS based oncology panels

Madhuri Hegde, PhD, FACMG
Professor
Executive Director, Emory Genetics Laboratory
Emory University



Ankala A, Hegde M, JMD, 2014



Williams E, Hegde M, Anat Path, 2014

NGS in Oncology

- NGS has advantages over traditional methods
- Ability to fully sequence large number of genes rather than “hot-spots”
- Simultaneous detection of
  - Single nucleotide variants (SNV), copy number alteration (CNA)-insertions, deletions and translocations
- Drug repurposing
  - Application of known/approved drugs to new indications/cancer type
  - New target discovery

Targeted Panels-Points to consider

- Capture method, efficiency and coverage
  - Overall and by gene
- Specimen type differences
  - Fresh-frozen vs. FFPE specimens
- Detection of single nucleotide variants (SNVs)
  - Methods
  - Filters
- Detection of indels and other mutation types
  - Methods- Experimental and informatics approaches

Advantages of detecting mutations with next-generation sequencing

- High throughput
  - >10^{11} base-pairs per run = test many genes at once
- Systematic, unbiased mutation detection
  - All mutation types (although some are more difficult than others)
- Digital readout of mutation frequency
  - Easier to detect and quantify mutations in a heterogeneous sample

Approaches to Validation

- Frequently involve the use of highly **heterogeneous** tissue specimens
- **Evidence based selection of targets-** clinically actionable somatic cancer mutations at low variant allele fraction (VAF)
- **VAF** for somatic tumor variants in the tissue specimen being tested depends on tumor cellularity
- How many samples? 20 or representative of all mutations included in the targeted assay tested multiple times- interrun and interpersonnel performances with varying VAF
- Reproduciblity studies
- Use of HapMap samples

*VAF refers to the fraction by which a certain variant at an allele is represented in the cell population within a tissue specimen

Approaches for Validation

- "Deep" sequence coverage (~1000 fold)
  - ***Reliable*** mutation detection (both known and novel)
  - Identification of mutations in heterogeneous specimens
- Unlike the case of constitutional variants, where the VAF is most often as high as 50% (heterozygous mutations; 100% for hemizygous or homozygous variants), the VAF for somatic pathogenic tumor variants or mutations can vary widely, frequently 20% or less.
- Strategic in silico mixtures of two different (Control) samples and assessing the analytic performance in detecting variants with a resultant VAF as low as 10% or less.
- Demonstrate 100% sensitivity for variants with VAF greater than 10% in the targeted regions
- Reproducibility and versatility of the assay by comparing results from different specimen types (formalin-fixed, paraffin-embedded tissue, fresh-frozen tissue, blood, bone marrow, and cell lines)

Advantages

- The relatively small target size of panels (compared to WES and WGS) allows for high coverage depth without significant increases in the sequencing cost
- In silico sensitivity analysis from many studies indicates that as high as 1000x unique coverage levels pick 100% of all variants with an allele fraction of at least 10%
- Detection of a high-quality, low-allelic fraction single nucleotide variant that was not detected by Sanger sequencing
- Detection of this true positive variant by a targeted NGS approach emphasizes the potential of the technology over the relatively less sensitive Sanger sequencing
- With optimal coverage depth and thorough validation of assays, the expectation is that NGS panels eventually can be offered as stand-alone tests without complementary Sanger confirmation
- Addition of new evidence based mutations to the panel

Limitations

- Lack of sufficient coverage in target exons - sensitivity of somatic variant detection.
  -Most assays can detect variants with an allele fraction as low as 10% with a sensitivity of 100%, the sensitivity for variants with allele fraction 5% and 1% is zero

- Knowing this is important, especially when dealing with impure and heterogeneous tumor specimens or with challenging specimen types, like formalin-fixed, paraffin-embedded samples, as formalin fixation is known to gradually degrade nucleic acids

- One added advantage of NGS assays, is their ability to process pooled multiple samples and perform parallel sequencing, allowing for an appreciable cost reduction.
  - The maximum number of samples that can be pooled together nevertheless is dependent on the size of the targeted region and the read and coverage depth required to make a confident call on the detected variant. However, this must be evaluated and validated for each individual NGS panel assay
  - Many studies have reported as low as 0.18% crossover during library preparation and 0.03% to 0.06% estimated crossover during multiplex sequencing of pooled samples

Eliezer Van Allen, MD

Dana Farber Cancer Institute

NGS panels and bioinformatics strategies for cancer applications

Eliezer (Eli) Van Allen, MD
Assistant Professor
Dana-Farber Cancer Institute
Broad Institute of MIT and Harvard
Harvard Medical School

February 25, 2016

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Outline

- Validation considerations for variant types
- Tumor-only panel testing considerations
- Inferring global genome properties from panels

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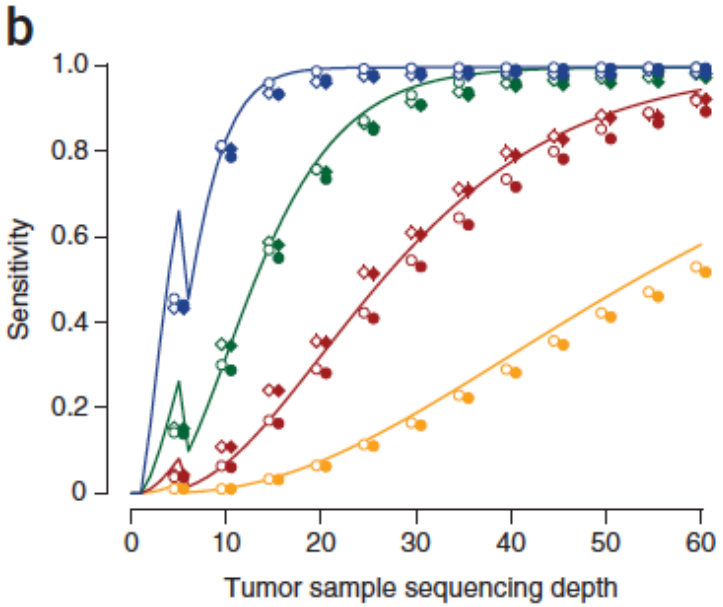
Somatic alteration detection

- Depending on the assay, options to identify:
  - Point mutations
  - Short insertion/deletion events
  - Copy number alterations
  - Fusion products (RNA)
- Level of analytical validation variable for different components

Somatic mutation analysis



- Similar results with other well-validated mutation callers (i.e. SomaticSniper, Strelka)
- Still tied to upstream sample quality and sequencing depth
- Documentation of analytical strategy and panel of normals is key

Table 2 Published validation rates of calls made by previous versions of MuTect in coding region

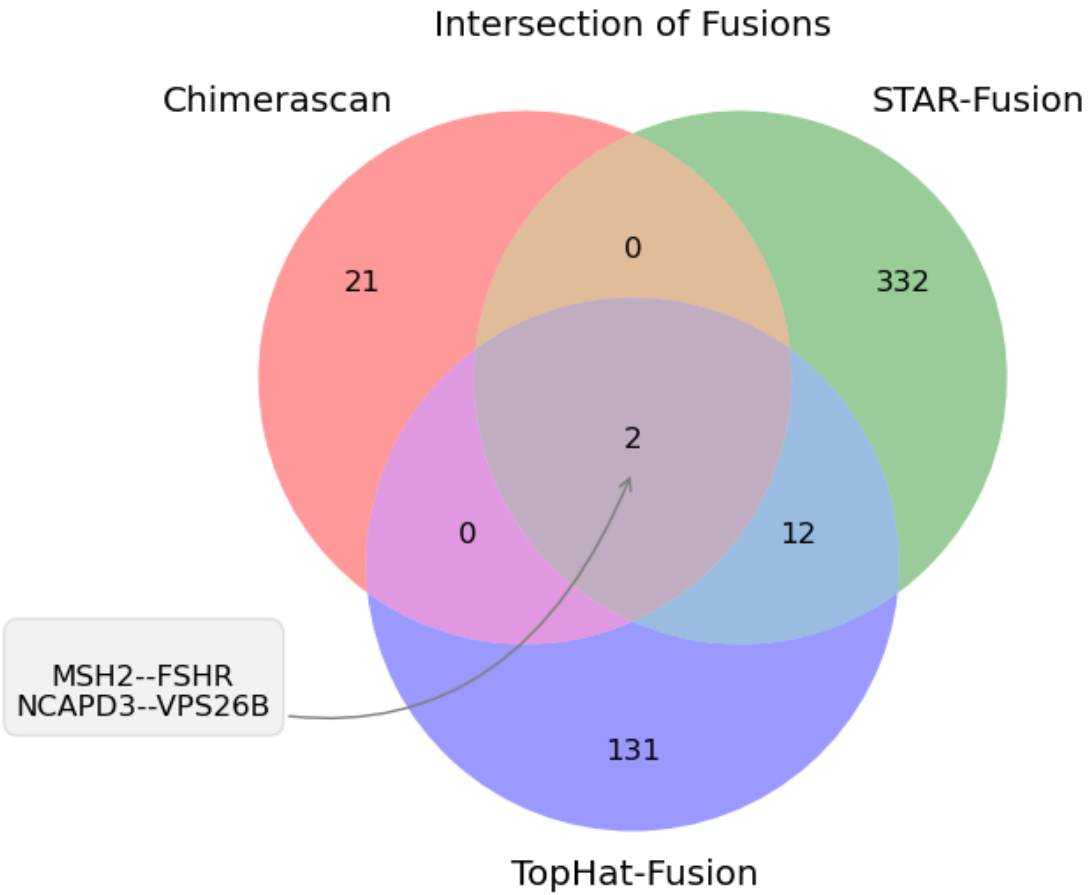
Tumor type	Mutation rate (Mb^{-1})	Validation technology	Validated	Invalidated	Validation rate (%)	False positive rate (Mb^{-1})
Multiple myeloma[19]	2.9	Sequenom	87	5	94.6	0.16
Head and neck[4]	3.3	Sequenom	181	8	95.8	0.14
Breast[3]	2.9	Sequenom/PCR/454	464	27	94.5	0.16
Prostate[24]	1.4	Sequenom	219	10	95.6	0.06
Colorectal[25]	5.9	Sequenom	292	16	94.8	0.31
CLL[26]	0.9	Sequenom	66	5	93.0	0.06
Medulloblastoma[27]	0.4[a]	Fluidigm/PacBio	19	0	100.0	NA (5 genes)
Prostate[28]	0.9	Sequenom	253	26	90.7	0.08
DLBCL[29]	3.2[a]	Fluidigm/Illumina	46	1	97.9	NA (6 genes)
TCGA colorectal[7]	14	PCR/454	5,713	420	93.1	0.96
Lung adeno[30]	12	Capture/Illumina	9,458	374	96.2	0.46

[a]Nonsilent.
NA, not applicable. CLL, chronic lymphocytic leukemia. DLBCL, diffuse large B-cell lymphoma. TCGA, The Cancer Genome Atlas. Adeno, adenocarcinoma.

Cibulskis et al *Nat Biotech* 2013; Ewing et al *Nat Methods* 2015

Somatic fusion detection

- RNA-based, but generalizable issue for other somatic alterations: indels, copy number, etc.
- Discrepancies between callers remains significant
- Orthogonal validation remains key need for some features

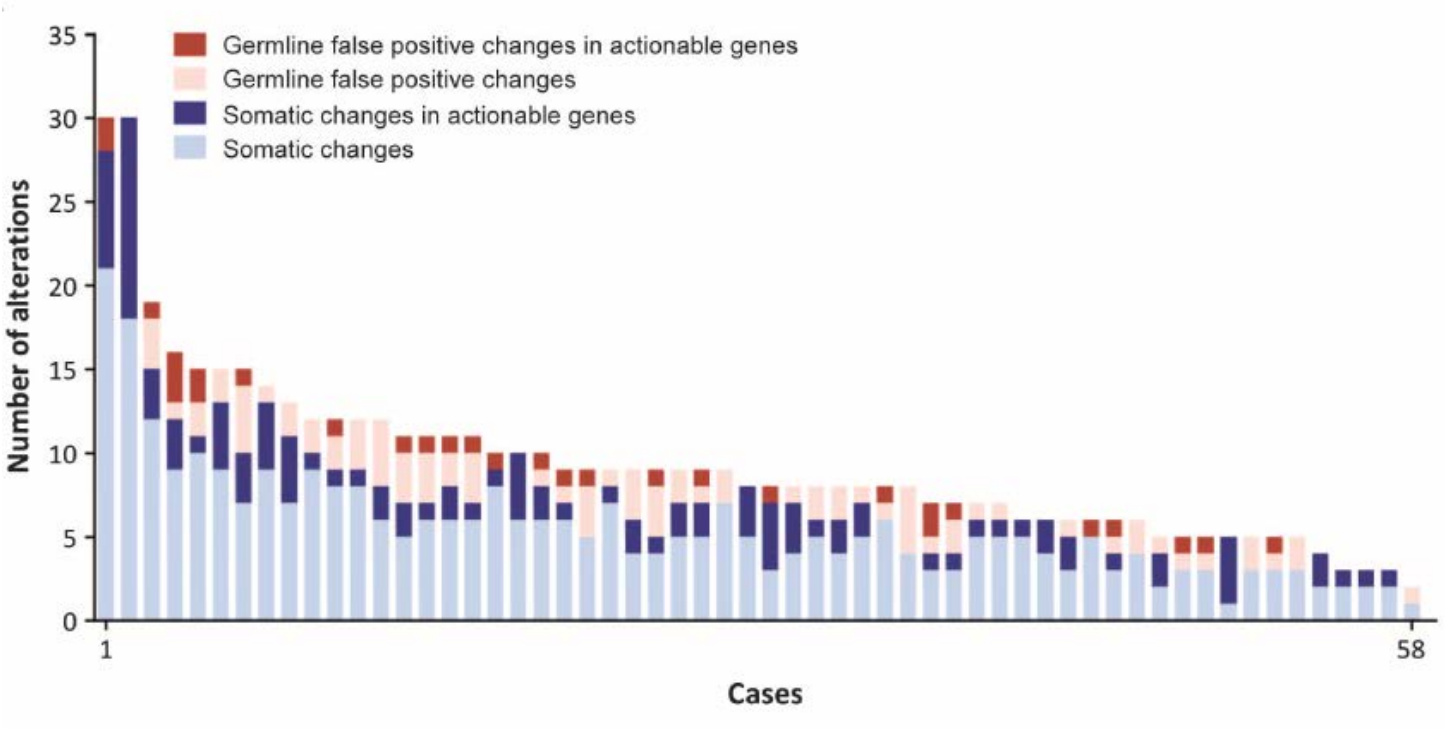


Outline

- Validation considerations for variant types
- Tumor-only panel testing considerations
- Inferring global genome properties from panels

Tumor only panel analysis

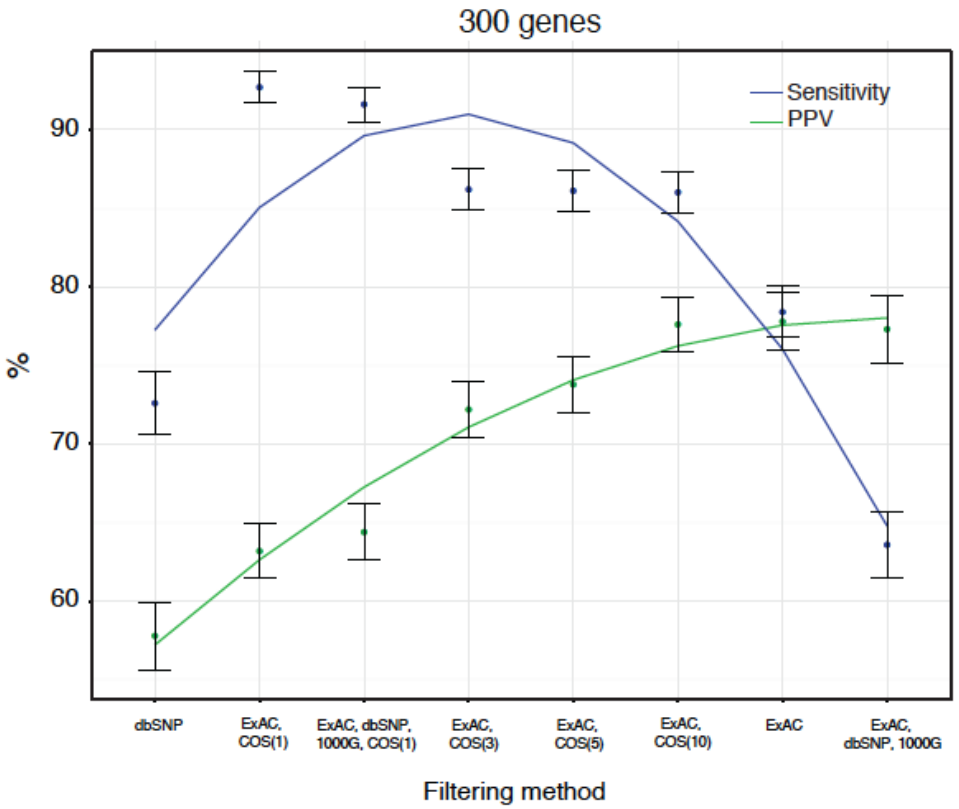
- Uncertainty about false positive germline variants
- Uncertainty about reporting pathogenic germline variants
- Disparities given imbalanced germline genome data?

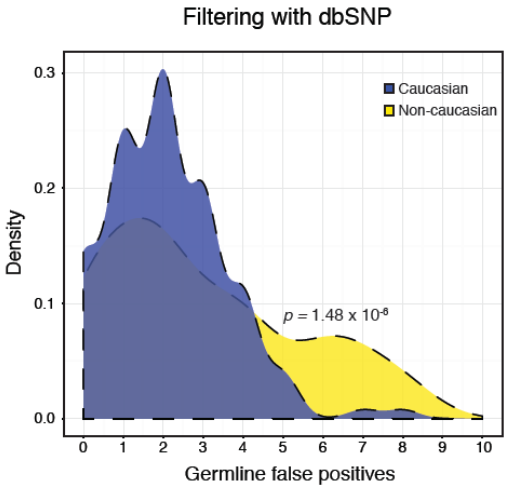


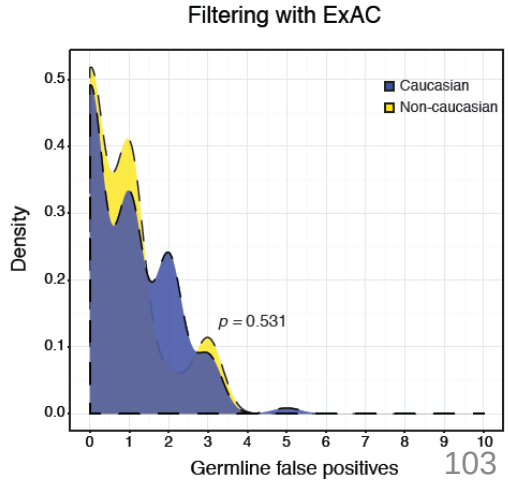
Jones et al *Science Trans Med* 2015; Schrader et al *JAMA Oncology* 2015

Tumor only panel analysis

- 157 clinical exomes
- Model tumor-only panels



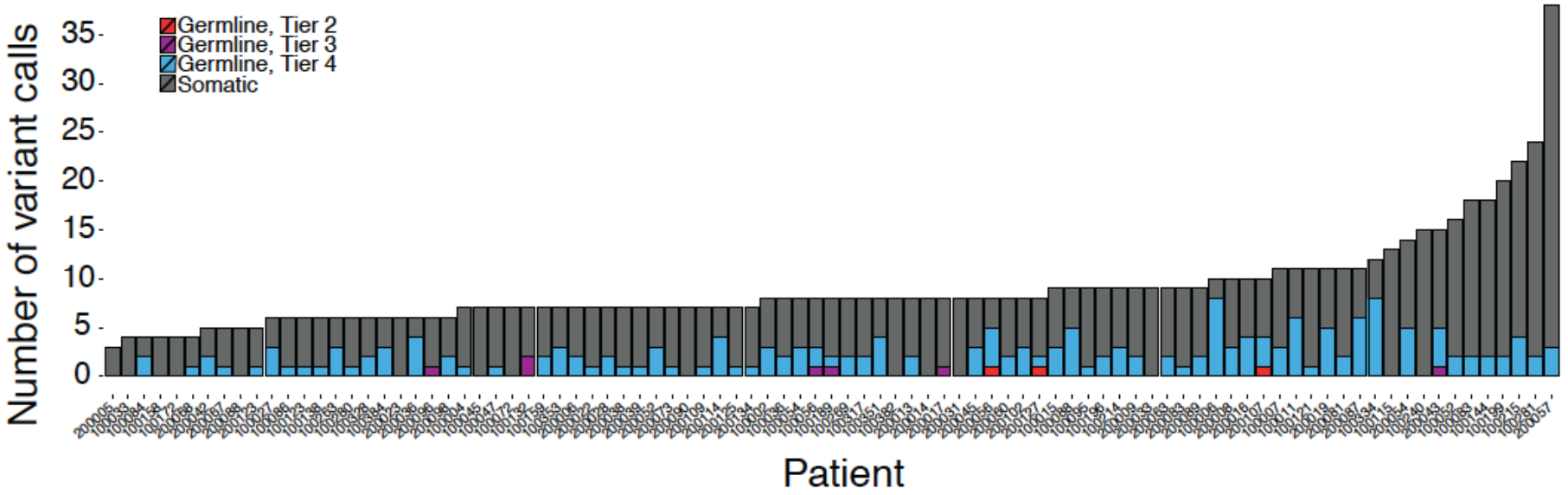




Andrea Garofalo

Role of molecular pathology in tumor-only analysis

- 14% germline false positive rate with large panels
- Downstream molecular pathology review correctly flagged 93% of these variants as likely germline
- Feature consideration for lab test procedures

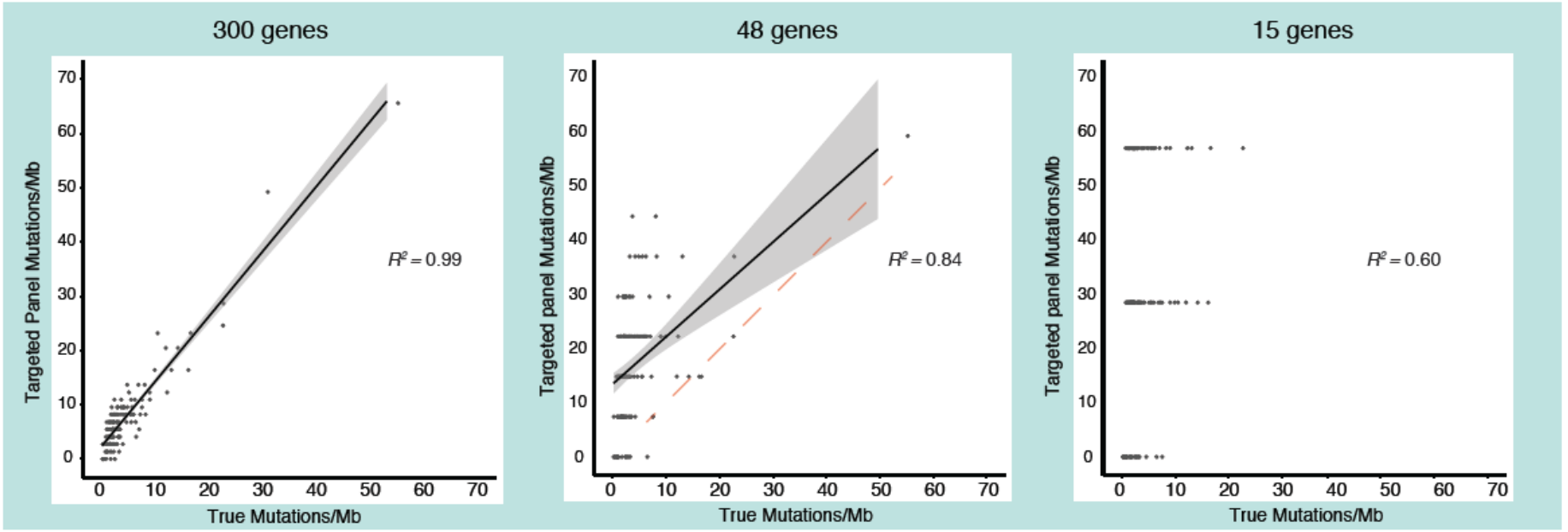


Andrea Garofalo, Lynette Sholl, Neal Lindeman, CAMD (BWH)

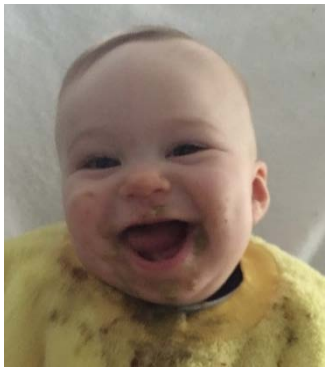
Outline

- Validation considerations for variant types
- Tumor-only panel testing considerations
- Inferring global genome properties from panels

Predicting genome-wide features



- Inferring ***mutational load*** from larger NGS panels feasible (matched or unmatched)
- Feasibility drops with smaller panel size
- Relevance for immuno-oncoogy applications and claims made for assay



Let's work together!

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Adam Keizun
Scott Carter
Will Gibson
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Ali Amin-Mansour
Many others…

DFCI + Center for Cancer Precision Medicine

Levi A. Garraway
Philip Kantoff
Mary-Ellen Taplin
Entire GU Oncology team
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Gregory Kryukov
Stacy Gray
Pasi Janne
Nikhil Wagle
Nelly Oliver
Karla Helvie
Anna Schinzel
George Demetri
Neal Lindeman
Lynette Sholl
Kwok-Kin Wong
David Barbie
Peter Hammerman
Many others…

The Patients

Funding

BroadIgnite

Panel Discussion Topic 2: Analytical Validation and Bioinformatics

Moderator: Donna Roscoe, Ph.D.

Panelists:

- **Madhuri Hegde, Ph.D. (Emory)**
- **Eliezer M. Van Allen, M.D. (Dana-Farber Cancer Institute)**
- **Josh Deignan, Ph.D. (UCLA)**
- **David Eberhard, M.D., Ph.D. (UNC, Chapel Hill)**
- **Robert Klees, Ph.D.(New York State Dept. of Health)**

Representative Variant Approach

FDA is seeking input as to how analytical performance with a representative variant set may be inferred for the entire panel.

- What types of considerations should weigh into the samples selected (and numbers) to obtain adequate and representative coverage, e.g., clinical relevance, number of variants/targeted regions reported, challenging parameters, platform bias? For different studies (e.g., accuracy, precision)?

- How should performance with indels be represented and evaluated? e.g., requirements for defined ranges (e.g., 0-10bp, 10-25bp), in each targeted region, in silico analysis

- Variant frequency (LoD for variant detection) can vary greatly; can evaluation of LoD for a variant type be representative of LoD performance for all variants of that type, or should LoD only be described for the specific variants evaluated?

Analytical Validation

FDA is seeking feedback on how assay performance can be objectively assessed.

- In your experience, what are acceptable orthogonal methods for accuracy and what considerations should go into confirming results so as to avoid bias in the assessment?
- How should assay sensitivity and specificity be defined in a meaningful way for end users and what is the most objective way to discuss performance? What are the critical performance metrics that should be assessed/reported and how should limitations/error rates for sequencing data be discussed?
- How should claims to distinguish somatic versus germline variants be validated?
- For what analytical tests should manufacturers begin at sample and end at assay report, and for what tests could the manufacturer start with nucleic acid, or even a BAM file?
- Should process controls be integrated into analytical validation testing?

Validating Modifications to the Panel

FDA is seeking input on what types of risk-based strategies could be used to ensure that minor panel modifications do not diminish assay performance.

- Once analytical validity has been satisfactorily established with a representative set of variants, what types of changes are not expected to change performance characteristics. Are there quality metrics that can be reported for determining if the change would impact assay performance?
  - Addition or subtraction of new variants and/or gene targets?
  - Different variant type (e.g., addition of CNV detection)?
  - Different variant size (large deletion)?
  - Change in variant calling requires a change in assay reagents?

- What risk-based strategies can be employed to determine when bioinformatics pipeline changes have a greater potential to impact assay performance?
  - What types of changes could be validated *in silico* using bioinformatics solutions and when would changes in the panel require running a sample set through the assay?

Panel Discussion Topic 2: Analytical Validation and Bioinformatics

Moderator: Donna Roscoe, Ph.D.

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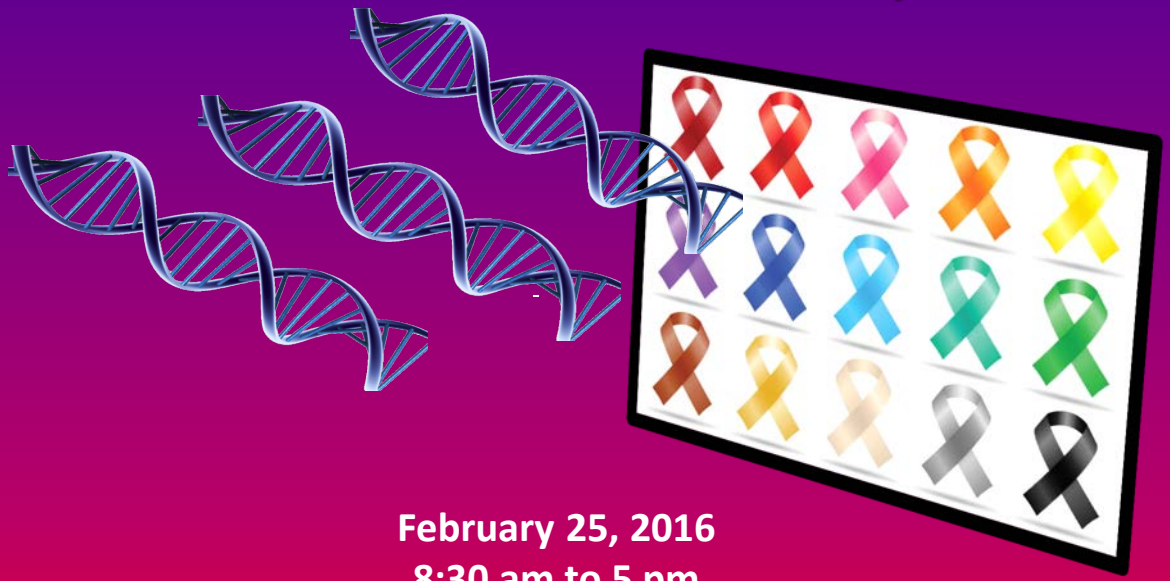
- **Robert Klees, Ph.D.(New York State Dept. of Health)**

LUNCH BREAK

12:30-1:30 pm

FOOD AND DRUG ADMINISTRATION

Next Generation Sequencing-based Oncology Panels Public Workshop



February 25, 2016
8:30 am to 5 pm

White Oak, MD

Webcast address: https://collaboration.fda.gov/ngsop0216/

FDA's Medical Devices News & Events Workshops & Conferences calendar:
http://www.fda.gov/MedicalDevices/NewsEvents/WorkshopsConferences/default.htm

Panel Discussion Topic 3:
Clinical and Follow-on Companion Diagnostic Claims

- **Moderator: Abraham Tzou, M.D.**
- **Panelists:**
  - **Shashi Kulkarni, Ph.D. (speaker)**
  - **Dane Dickson, M.D. (speaker)**
  - **Gideon Blumenthal, M.D.**
  - **Greta Kreuz (patient advocate)**
  - **Jeffrey Sklar, M.D., Ph.D.**
  - **Apostolia-Maria Tsimberidou, M.D., Ph.D.**

Shashi Kulkarni, Ph.D.

Washington University

Clinical Cancer Genomics

Shashi

Shashikant Kulkarni, M.S (Medicine)., Ph.D., FACMG
Director of Cytogenomics and Molecular Pathology
Professor of Pediatrics, Genetics, Pathology and Immunology

GENOMICS AND PATHOLOGY SERVICES
GPS@WUSTL
CLINICAL TESTING • RESEARCH • EDUCATION

Washington University Physicians
Washington University School of Medicine in St. Louis

Children's HOSPITAL • ST. LOUIS

BARNES-JEWISH Hospital
BJC HealthCare

SITEMAN CANCER CENTER
BARNES-JEWISH HOSPITAL • WASHINGTON UNIVERSITY SCHOOL OF MEDICINE
A National Cancer Institute Comprehensive Cancer Center

Disclosures

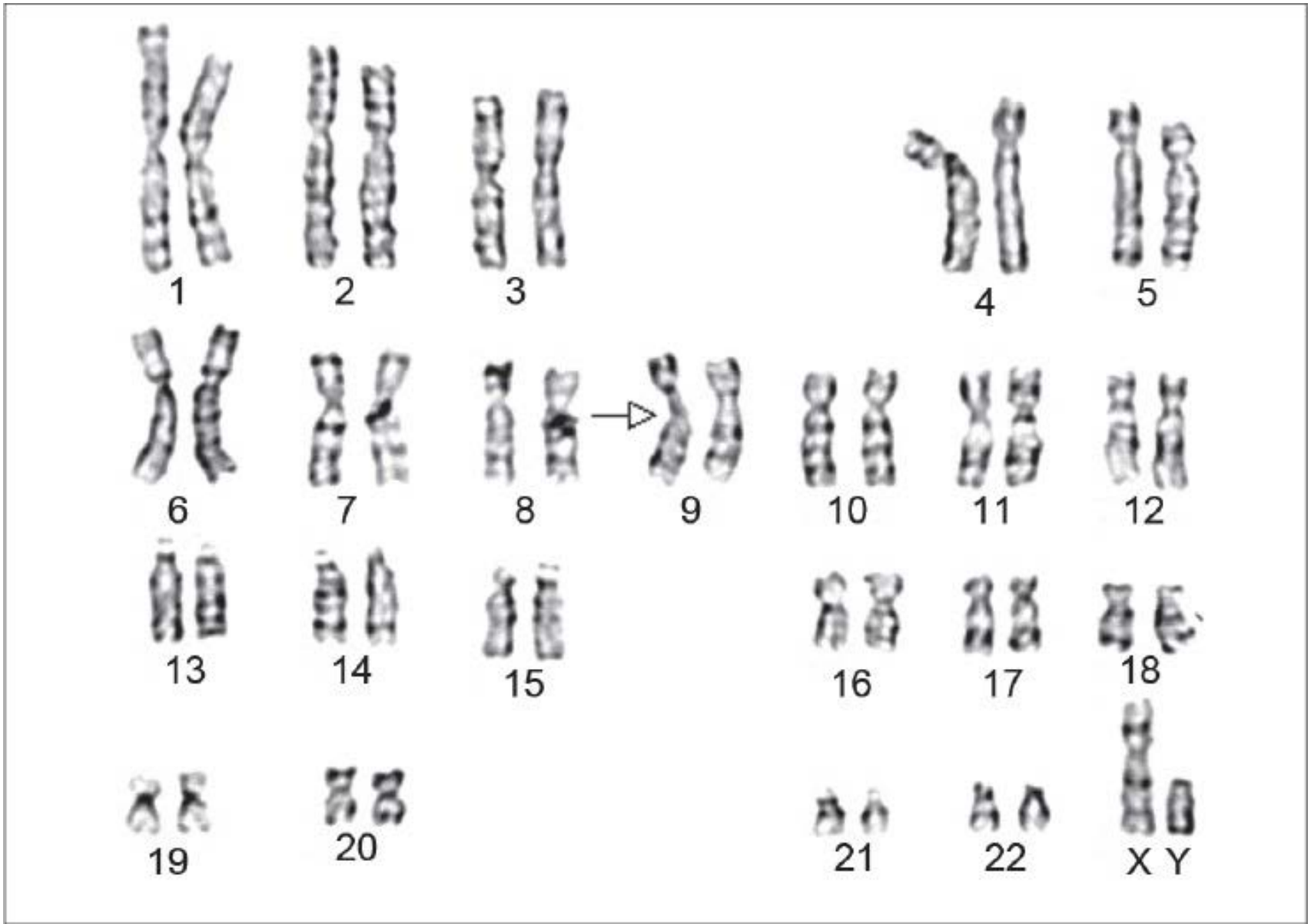
- **Professor of Pathology & Immunology and Director of Cytogenomics and Molecular Pathology (generates revenue)**
- Scientific Advisory Board
  - National Institute of General Medical Sciences (NIGMS)
  - Princess Margaret Cancer Center, Toronto
  - Swift Biosciences
  - Bina Technologies
  - Horizon discovery
- Editorial honorarium
  - Cancer Genetics (journal); Elsevier (Ref module and book editor)
- Speaker honorarium
  - American College of Medical Genetics and Genomics (ACMG), National Cancer Institute (NCI), Association of Molecular Pathology (AMP), Illumina, Novartis, Agilent, Biodiscovery, Affymetrix

CLINICAL NGS FOR CANCER DIAGNOSTICS

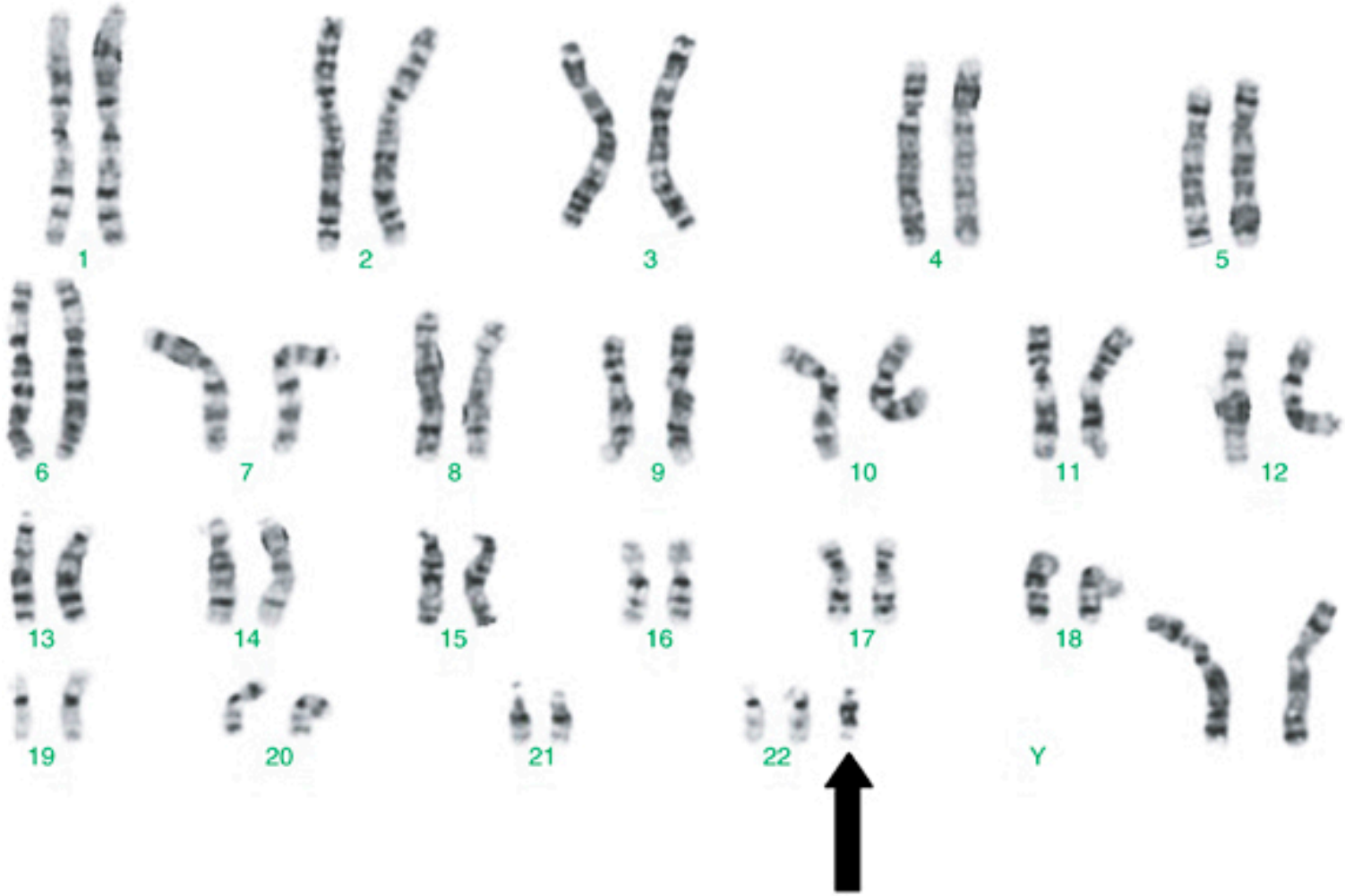
Determination of Clinical Significance of Variants

Variant interpretation issues are not new!

Chromosome 9 variant (benign)



Extra marker chromosome (pathogenic)



47,XX,+mar.ish psudic(22;22)(q11.2q11.2
D14Z1/D22Z1++,D22Z4++,D22S75-)

Variant rating system

CLINICAL GENETICS An International Journal of Genetics, Molecular and Personalized Medicine

Clin Genet 2012: 81: 403–412

CLINICAL GENETICS
doi: 10.1111/j.1399-0004.2011.01818.x

Review

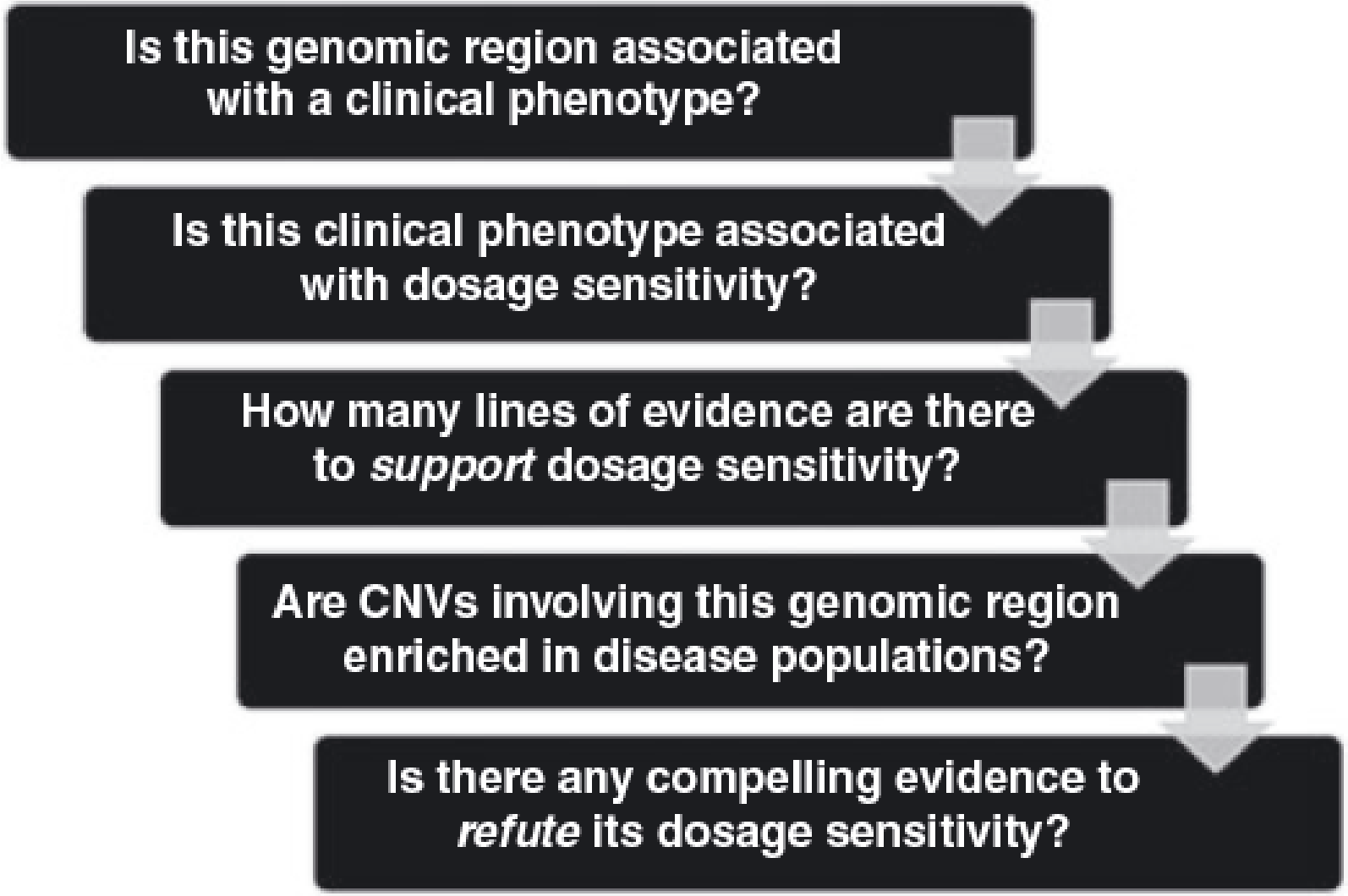
Towards an evidence-based process for the clinical interpretation of copy number variation

Riggs ER, Church DM, Hanson K, Horner VL, Kaminsky EB, Kuhn RM, Wain KE, Williams ES, Aradhya S, Kearney HM, Ledbetter DH, South ST, Thorland EC, Martin CL. Towards an evidence-based process for the clinical interpretation of copy number variation.
Clin Genet 2012: 81: 403–412. © John Wiley & Sons A/S, 2011

The evidence-based review (EBR) process has been widely used to develop standards for medical decision-making and to explore complex

ER Riggs[a], DM Church[b], K Hanson[c*], VL Horner[a], EB Kaminsky[a], RM Kuhn[d], KE Wain[e], ES Williams[a], S Aradhya[f], HM Kearney[g], DH Ledbetter[h], ST South[i], EC Thorland[g] and CL Martin[a,*]

Framework for evidence-based process



Clinical Evidence process of actionability

- Lead to changes in the clinical management of patients
- Predict survival or other clinical end points independent of any specific treatment (**prognostic**)
- Predict response to treatment (**therapy-guiding or predictive**)
- Assess response to treatment (“monitoring”)
- Identify the risk of organ-based toxicities or altered metabolism and/or response to cancer drugs (**pharmacogenomic**)

External databases and tools

Databases relevant to interpretation of somatic sequence variants

Database	Location (URL)
Population databases to exclude polymorphisms	
1000 genomes project	http://browser.1000genomes.org
Exome Variant Server	http://evs.gs.washington.edu/EVS
dbSNP	http://www.ncbi.nlm.nih.gov/snp
dbVar	http://www.ncbi.nlm.nih.gov/dbvar
ExAC	http://exac.broadinstitute.org/
Cancer specific variant databases	
Catalogue of Somatic Mutations in Cancer (COSMIC)	http://cancer.sanger.ac.uk/cosmic
My Cancer Genome	http://www.mycancergenome.org/
Personalized Cancer Therapy, MD Anderson Cancer Center	https://pct.mdanderson.org/
cBioPortal, Memorial Sloan Kettering Cancer Center	http://www.cbioportal.org/
Intogen	https://www.intogen.org/search
ClinicalTrials.gov	https://clinicaltrials.gov/
IARC (WHO) TP53 mutation database	http://p53.iarc.fr/

Databases relevant to interpretation of somatic sequence variants

Database	Location (URL)
Pediatric Cancer Genome Project, St Judes Children's research Hospital and WashU joint venture	http://explorepcgp.org/
International Cancer Genome Consortium (ICGC)	https://dcc.icgc.org/
Sequence repositories and data hosts	
NCBI Genome	http://www.ncbi.nlm.nih.gov/genome
RefSeqGene	http://www.ncbi.nlm.nih.gov/refseq/rsg
Locus Reference Genomic (LRG)	http://www.lrg-sequence.org
UCSC table browser	https://genome.ucsc.edu/cgi-bin/hgTables
Ensemble BioMart	http://useast.ensembl.org/biomart/martview/
Other Disease/Mutation databases useful in the context of variant interpretation for cancer genomics	
ClinVar	http://www.ncbi.nlm.nih.gov/clinvar
Human Gene Mutation Database	http://www.hgmd.org
Locus Reference Genomic (LRG)	http://www.lrg-sequence.org
Leiden Open Variation Database	http://www.lovd.nl
dbNSFP (compiled database of precomputed in-silico prediction scores for non-synonymous	https://sites.google.com/site/jpopgen/dbNSFP

Algorithms for computational prediction of functional impact of sequence variant / splice site changes

Algorithm / Software	Location (URL)
PolyPhen2	http://genetics.bwh.harvard.edu/pph2
SIFT	http://sift.jcvi.org
MutationAssessor	http://mutationassessor.org
MutationTaster	http://www.mutationtaster.org
PROVEAN	http://provean.jcvi.org/index.php
Condel	http://bg.upf.edu/condel/home
CADD	http://cadd.gs.washington.edu
GERP	http://mendel.stanford.edu/sidowlab/downloads/gerp/index.html
PhyloP	http://compgen.bscb.cornell.edu/phast/
PhastCons	http://compgen.bscb.cornell.edu/phast/
Splice site Prediction	
Human Splicing Finder	http://www.umd.be/HSF
MaxEntScan	http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html
NetGene2	http://www.cbs.dtu.dk/services/NetGene2
NNSplice	http://www.fruitfly.org/seq_tools/splice.html
GeneSplicer	http://www.cbcb.umd.edu/software/GeneSplicer/

Clinical Evidence process of actionability

- Lead to changes in the clinical management of patients
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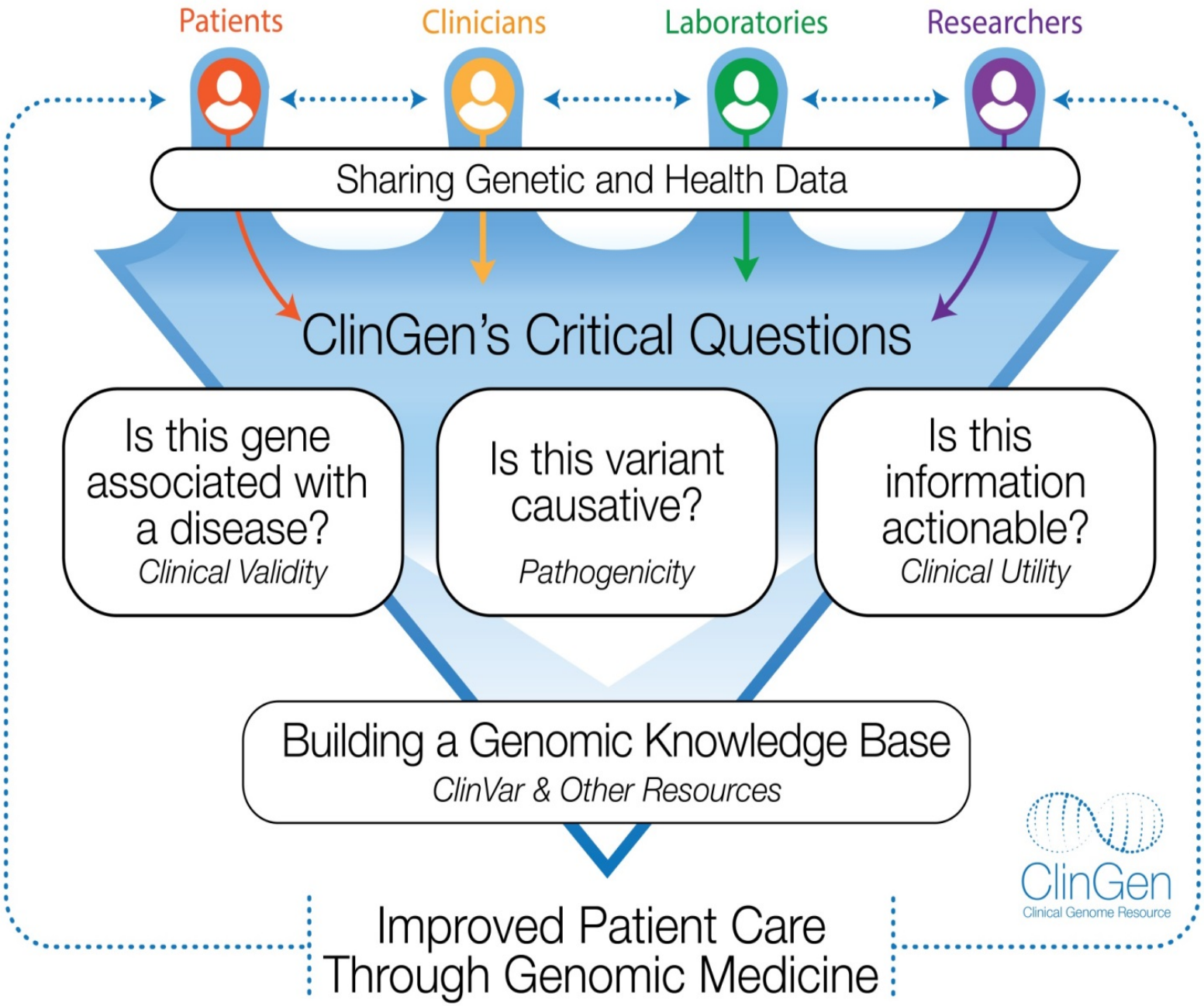
- Predict response to treatment (**therapy-guiding or predictive**)
- Assess response to treatment (“monitoring”)
- Identify the risk of organ-based toxicities or altered metabolism and/or response to cancer drugs (**pharmacogenomic**)

WU Somatic Variant Classification

- Level 1- Predictive or prognostic in tumor type
  - Example *BRAF* V600E in melanoma
- Level 2- Predictive or prognostic in other tumor type(s)
  - *IDH1* R132 in colon cancer
- Level 3- Reported in cancer or other disease
  - Reported in COSMIC, TCGA, etc. and documented as cancer-associated but with no known clinical significance
- Level 4- Variant of uncertain significance
  - Not in COSMIC, TCGA, etc and MAF < 1% in population database (ESP, ExAC, or 1000G)
- Level 5- Known polymorphism
  - Variant in population database (ESP, ExAC, or 1000G) with MAF>1%

Challenges and Opportunities

- We are witnessing a paradigm shift in clinical cancer genomics
- **Building of cancer variants knowledge network by experts is very important**
- NIH funded ClinGen Resource
  - Gathers and curates data about the strength of relationships among genes, variants, and diseases
  - Somatic workgroup established


Patients
Clinicians
Laboratories
Researchers
Sharing Genetic and Health Data
ClinGen's Critical Questions
Is this gene associated with a disease?
Clinical Validity
Is this variant causative?
Pathogenicity
Is this information actionable?
Clinical Utility
Building a Genomic Knowledge Base
ClinVar & Other Resources
ClinGen
Clinical Genome Resource
Improved Patient Care Through Genomic Medicine

ClinGen Somatic Working Group

- **Vision:** The Cancer Somatic Workgroup aims to collaborate with expert groups to ***develop processes that support accurate determination of clinical relevance of somatic changes for use by physicians, clinical laboratories, researchers, and guideline-developing groups***.

- **Mission:** The mission of the Cancer Somatic Workgroup is to ***facilitate the development of standards for classification and interpretation of somatic changes and their clinical actionability in order to enhance the usability, dissemination and implementation of cancer somatic changes*** in the ClinGen resource to enable implementation of precision cancer care.

Team

- Medical oncologists, molecular pathologists, clinical genomics lab directors and informaticians from **over 50** academic cancer research organizations and industry partners
- Multi-Disciplinary Team led by
  - *Shashikant Kulkarni, PhD., FACMG* - Director, Cytogenomics and Molecular Pathology, Professor, Washington University School of Medicine
  - *Subha Madhavan, PhD* - Director, Innovation Center for Biomedical Informatics, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center
  - *Sameek Roychowdhury, MD, PhD* - Medical Oncologist and Clinical Researcher, Medical Director, CLIA Cancer Genomics Lab, The James Cancer Hospital and Comprehensive Cancer Center, Ohio State University
  - *Eliezer Van Allen, MD* – Medical Oncologist and Clinical Researcher, Computational Director, Center for Cancer Precision Medicine, Dana Farber Cancer Institute & Harvard Medical School

Somatic Cancer Co-chairs

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Washington University School of Medicine

Subha Madhavan, PhD
Georgetown University

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National Cancer Institute

Christine Walko, PharmD
Moffitt Cancer Center

Mike Watson, PhD
ACMG

Not Pictured:
Annette Meredith, PhD, MPL
Jason Merker, MD, PhD, Stanford

There are many working groups for clinical cancer genomics

CSER Tumor Working Group
Approaches for adapting genomics in the clinic

Association for Molecular Pathologists (AMP)
Guidelines for somatic variant interpretation

Global Alliance for Genomics and Health (GA4GH)
Data sharing; strong in data “representation”

GENIE: Real time CLIA data and outcomes
7 institutions

Actionable Cancer Genome Initiative (ACGI)
4 institutions + Illumina, best practices

Others, Private or commercial efforts

QUESTIONS and NEEDS for What and How?

- Data Sharing
- Common Language
- Guidelines for Classification
- Guidelines for Interpretation
- Guidelines for new test development

What is the CLINGEN Somatic working group working on

Leverage experiences of clinicians and lab directors to develop data elements for "presentation" of data to aid in somatic variant classification and clinical actionability

Current activities

Define Common Language for biomarkers using controlled vocabularies

Define Minimum Variant Level Data (MVLD)

Define Minimum Case Level Data (MCLD)

Minimal Variant Level Data

ClinGen
Clinical Genome Resource

Data Element	DESC	EXAMPLE	Acceptable Formats	ClinVar (does it have this field)
Genome Version	the reference genome used for making the variant call. Use GRChXX version, and if possible use specific version data was aligned to.	GRCh37.2	hg19, hg18, GRCh37, not null	YES (assembly location provided, unknown on what assembly variant was called)
Gene	the reference HUGO gene name	BRAF	HUGO name or null (noncoding) *[Will accept other formats?]*	YES
Chromosome	reference chromosome	7	7, chr7 (like UCSC bed file?), not null	YES
DNA Position	reference genomic position	140453135	not null	YES
Refseq Transcript	the reference transcript (Refseq gene name)	NM_004333.4	NM_XXXXXX.X or NM_XXXXX (*not null?*)	YES (Yes, multiple transcript IDs)
Refseq Protein	the reference protein ID	NP_004324	NP_XXXXXX or null (wil accept without?)	YES (multiple protein IDs)
DNA Substitution & Position	human genome variation standard formatting for genomic variant	c.1799T>A	HGVS [what about noncoding? Null here?]	YES
Protein Substitution & Position	human genome variation standard formatting for protein variant	p.V600E	HGVS [what about noncoding? Null here?]	YES
Mutation Type	The effect of the mutation [nonsense, missense, silent, etc]	Missense	[need discussion of allowable descriptors here: Stop, Nonsense, Missense, Frame Shift, Stop Loss, Stop Gain, Noncoding Regulatory etc]	YES
Cancer Type	cancer type for which the clinical interpretation is relevant	Melanoma	See F11	YES (but not required to fill. Ex "not provided" is an example of the "condition" field for TP53 somatic variant in ClinVar
Level of Evidence	a score assessing the evidence of the role the variant plays in conferring the cancer phenotype *[DISCUSSION TOPIC: THE LEVELS OF EVIDENCE FROM CANDL AS A STARTING POINT:* *Alteration has matching FDA approved or NCCN recommended therapy.* *Alteration has matching therapy based on evidence from clinical trials, case reports, or exceptional responders.* *Alteration predicts for response or resistance to therapy based on evidence from pre-clinical data (in vitro or in vivo models).* *Alteration is a putative oncogenic driver based on functional activation of a pathway.]*	ranked descriptive statement and numerical score	See F12	YES (a review status and clinical significance limited language descriptors)
PMIDs	literature citing the variant, functional evidence and involvement with cancer	12068308, 21639808	Pubmed ID or null	YES
Biomarker Class	a keyword description related to the clinical utility of the variant	Diagnostic, Prognostic, Predictive	See F14	NOT EXACTLY (the clinical significance is closest, but does not use these keywords)
Therapeutic Context	known associated drugs for this variant. Dienstmann et al also uses descriptions for broad classes of inhibitors such as "PI3K inhibitors" etc	Vemurafenib, Dabrafenib	See F15	NO
Effect	Keywords describing the effect of the variant in the therapeutic context [keywords from Dienstmann et al are: resistant, responsive, not-responsive, sensitive, reduced sensitivity]	Responsive	See F16	NO
Sub-Level of Evidence	matches "Status" and *"Level of Evidence"* from Dienstmann, and would include:Prospective trials, retrospective trials,expert opinion, case reports, preclinical, inferential *[Discussion Topic: IN SOME INSTANCES, RESPONSIVE AND RESISTANCE ARE NOT QUITE BLACK AND WHITE, while some are. E.G. BRAF V600E IS NON-RESPONSIVE FOR HALF OF PATIENTS, BUT RESPONSIVE FOR OTHER HALF. EGFR T790M IS NON-RESPONSIVE FOR ERLOTINIB, BUT COULD BE RESPONSIVE FOR 3RD GENERATION DRUG AT X% (TBD)]*	RCT, Expert opinion	See F17	NOT EXACTLY (review status/assertion method) is closest, but can be blank.

Active collaboration discussions

- ClinVar
  - Infrastructure for curating variants
- Mycancergenome
  - Medical interpretation curation methodology
  - Potential pilot for Melanoma-BRAF variants
- AACR GENIE
  - Clinical outcomes
- GA4GH/Actionable Cancer Genome Initiative
  - Infrastructure
  - Pilot projects for data sharing
- ACMG
  - Variant classification standards
- CSER
  - Data sharing
- **AMP**
  - **Guidelines for Variant classification**

Future activities

- Continue to define standards for capturing and sharing somatic variant data to aid in classification and medical interpretation
- Support Somatic Data Curation, interpretation and sharing
- Identify appropriate technology infrastructure for data capture and sharing

Upcoming events

- Second face to face discussion
  - April 17; 4 to 8 PM @AACR, New Orleans
- GA4GH data sharing meeting
  - April 15 @AACR, New Orleans
- Informal discussion of WG members
  - @ACMG, Tampa

ClinGen Acknowledgements

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Robert Nussbaum
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Michael Watson

Erica Anderson
Swaroop Arahdya
Sandy Aronson
Euan Ashley
Larry Babb
Erin Baldwin
Sherri Bale
Louisa Baroudi
Les Biesecker
Chris Bizon
David Borland
Rhonda Brandon
Michael Brudno
Damien Bruno
Atul Butte
Hailin Chen
Mike Cherry

Soma Das
Johan den Dunnen
Edwin Dodson
Karen Eilbeck
Marni Falk
Andy Faucett
Xin Feng
Mike Feolo
Matthew Ferber
Penelope Freire
Birgit Funke
Monica Giovanni
Katrina Goddard
Robert Green
Marc Greenblatt
Robert Greenes
Ada Hamosh
Bret Heale
Madhuri Hegde
Ray Hershberger
Lucia Hindorff
Sibel Kantarci
Hutton Kearney
Melissa Kelly
Muin Khoury
Eric Klee
Patti Krautscheid
Joel Krier
Danuta Krotoski
Shashi Kulkarni
Matthew Lebo
Charles Lee

Jennifer Lee
Elaine Lyon
Subha Madhavan
Teri Manolio
Rong Mao
Daniel Masys
Peter McGarvey
Dominic McMullan
Danielle Metterville
Laura Milko
David Miller
Aleksander Milosavljevic
Rosario Monge
Stephen Montgomery
Michael Murray
Rakesh Nagarajan
Preetha Nandi
Teja Nelakuditi
Annie Niehaus
Elke Norwig-Eastaugh
Brendon O'Fallon
Kelly Ormond
Daniel Pineda-Alvaraz
Darlene Reithmaier
Erin Riggs
George Riley
Peter Robinson
Wendy Rubinstein
Shawn Rynearson
Cody Sam
Avni Santani

Neil Sarkar

Melissa Savage
Jeffery Schloss
Charles Schmitt
Sheri Schully
Alan Scott
Chad Shaw
Weronika Sikora-Wohlfield
Bethanny Smith Packard
Tam Sneddon
Sarah South
Marsha Speevak
Justin Starren
Jim Stavropoulos
Greer Stephens
Christopher Tan
Peter Tarczy-Hornoch
Erik Thorland
Stuart Tinker
David Valle
Steven Van Vooren
Matthew Varugheese
Yekaterina Vaydylevich
Lisa Vincent
Karen Wain
Meredith Weaver
Kirk Wilhelmsen
Patrick Willems
Marc Williams
Eli Williams

Dane Dickson, M.D.

Molecular Evidence Development Consortium

MED-C

Molecular Evidence Development Consortium

FDA – Public Workshop
NGS Panels In Oncology

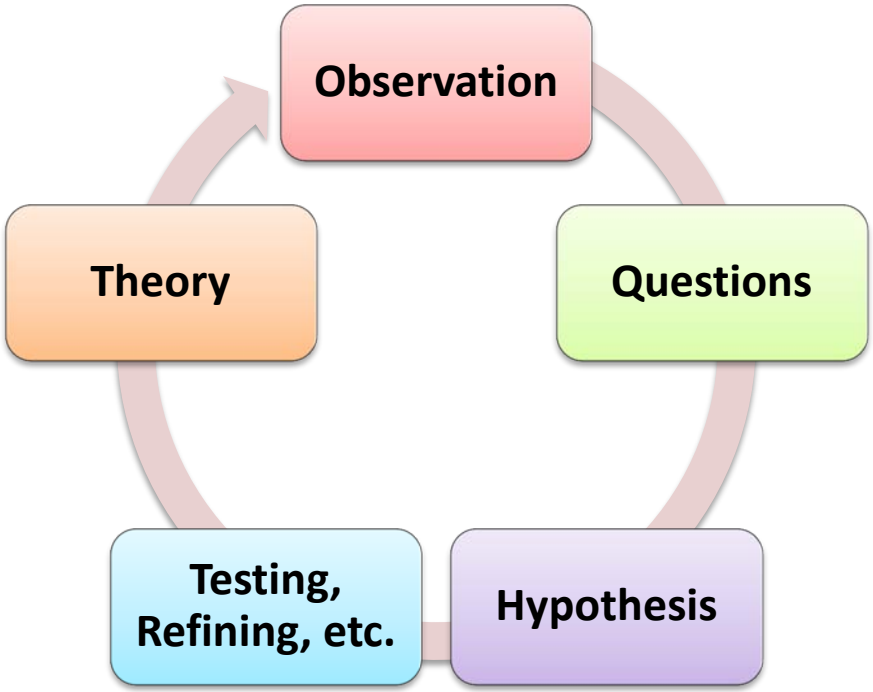
Dane J. Dickson MD

CEO Molecular Evidence Development Consortium

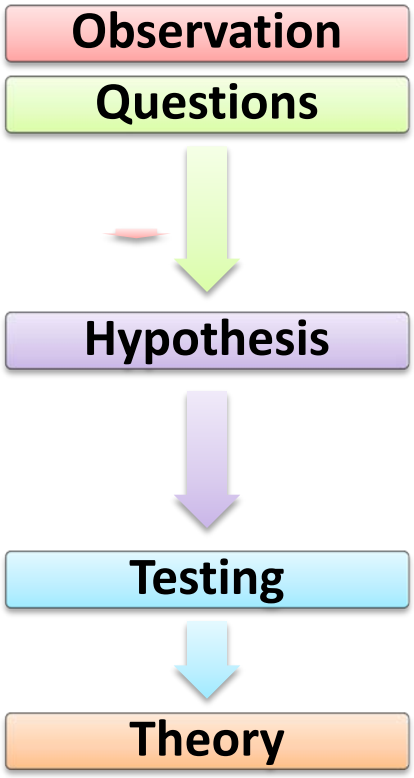
Director of Precision Medicine Policy and Registries | Oregon Health and Science University

Essential Tenants

- Science is Science – and although we have new technology and new applications, we need to remember the scientific method



NGS and Scientific Method



Current State of Affairs:

- **Observation/Questioning**: NGS is a "Swiss Army Knife" of testing (?) OR (.) OR (!)
  - **May possibly do the work of** Sanger, IHC, FISH, PCR, etc.
  - Less tissue needed
  - May be at lower cost
- **Hypothesis:** NGS **Can** Replace **(and may be better than)** other testing
  - Companion Diagnostics (CDx)
  - Other biomarkers

Where we Need to Go:

- **Testing:**
  - How to we show analytical equivalence (or superiority)
  - How to we show clinical equivalence (or superiority)
- **Theory:**

Usual Testing Methods

Define Test	• Determine Type of Testing • Establish Analytical Validity
Define Patients	• Indicated Usage • **Clinical Validity** of Test
Define Intervention based on Test	• Test if positive leads to action • Action needs to be predefined
Collect Outcomes	• Show that the test lead to better outcome **(Clinically Utility)**

Define NGS Testing

Pre-Analytic	Sequencing	Informatics Pipeline
Hybrid Capture	Instrument 1	Algorithm 1
Amplification	Instrument 2	Algorithm 2
Version and Variation	Instrument X	Algorithm X
	Sequencing Type	Version and Variation
	Biomarker Panel	
	Depth of Read	
	Version and Variation	

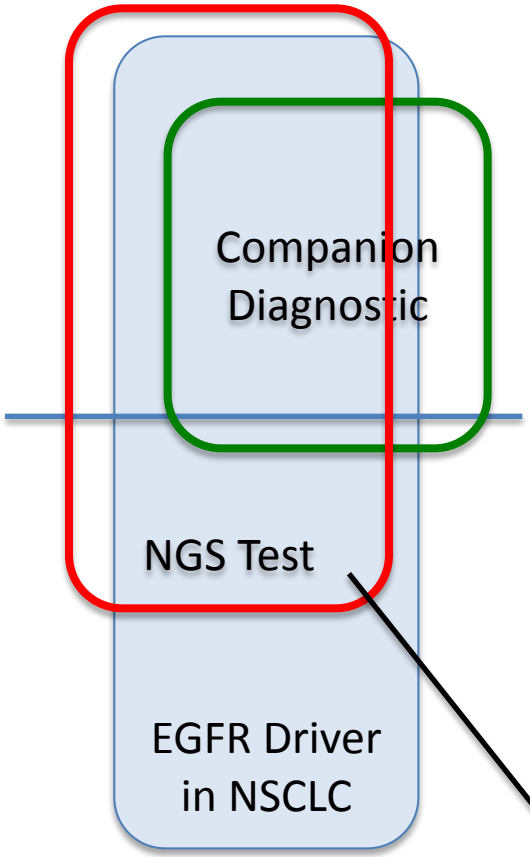
How to think about each test (Somatic Tumor):

$PA(H_{1.6})$ $SQ(IL_{3.1},WE_{2.2},P_4)$ $IP(ABC_{1.2})$

$PA(A_{2.1})$ $SQ(TF_{2.6},TE_{3.0},P_3)$ $IP\ (XYZ_{2.3})$

Testing

Analytic Differences can be huge



Clinical Threshold For Benefit

- Increased sensitivity in
  - A) Picking up different alterations
  - B) Picking up lower allele frequency
- May not result in better overall outcomes
- THEREFORE NEED CLINICAL OUTCOMES TO DETERMINE OVERALL BENEFIT

$NGS[PA(H_{1.6})\ SQ(IL_{3.1},WE_{2.2},P_4)\ IP(ABC_{1.2})]$

Trial Designs

Do we need trials?

Endpoints? Analytical or Clinical?

Can we simplify anything?

- Phase III RCT – Standardized NGS vs. CDx, looking at overall RR, TTP and OS by biomarker detected both methods
- Comparative Trials – Two way comparison
  - Biomarker by CDx also tested by standardized NGS (I.e. NCI-MATCH or MED-C Registry)
  - Biomarker by NGS compared to CDx
  - Both looking at clinical outcome
- Retrospective Analysis – Data review
  - Archived tissue - +, retest using NGS – show general equivalence
- General Consensus of a "Standard of Care without Published Data"
  - Already taking place in many institutions – is this okay?
  - Make some assumptions (standardized NGS can replace CDx) and collect outcomes from these patients based on NGS and see how compare to expectations based on previous CDx clinical trials
  - Very important to collect outcomes, especially when dealing with 100s of analytes and could easily see complex mutations

Dickson's Perspective

Do we need trials?

-Absolutely

With the binary therapy decision of many biomarkers – we need to know who we are helping or hurting.

End points of Trials?

-Clinical

The increased sensitivity of NGS, the broad nature of panels, the confusion of multiple biomarkers, etc.

Simplify/Standard of Care without Published Data?

-Maybe, BUT

a) Standardize NGS testing (and figure out which

$$NGS[\ PA(Type_{a.a})\ SQ(Instrument_{b,b}, AnalysisType_{c.c}, Panel_{d})\ IP(Developer_{e,e.})\ ]s$$

are equal)

b) Collect outcomes on ALL patients (No N of 0 Experiments) in a shared, non-proprietary, research open access database

Panel Discussion Topic 3:
Clinical and Follow-on Companion Diagnostic Claims

- **Moderator: Abraham Tzou, M.D.**
- **Panelists:**
  - **Shashi Kulkarni, Ph.D. (speaker)**
  - **Dane Dickson, M.D. (speaker)**
  - **Gideon Blumenthal, M.D.**
  - **Greta Kreuz (patient advocate)**
  - **Jeffrey Sklar, M.D., Ph.D.**
  - **Apostolia-Maria Tsimberidou, M.D., Ph.D.**

Follow-on Companion Diagnostics

FDA generally asks for clinical data for follow-on companion diagnostics because failure to select the same patient population could lead to changes in treatment outcome.

- In your expert opinion, what level of agreement would be sufficient to provide a reasonable assurance of safety and effectiveness for a follow-on companion diagnostic?

- What would be good clinical sample sources? Please keep in mind that the original clinical trial samples (which would be ideal) are usually not available. Please consider procured specimens to mirror the therapeutic patient population and differences in specimen type (e.g., the companion diagnostic used FFPE but the NGS panel utilizes fresh frozen tissue).

Non-Companion Diagnostics

FDA is interested in input on the level of clinical validity that should be established for any variant reported by the assay that does not have a companion diagnostic claim.

- What are your thoughts about the inclusion of variants based on the establishment of comparable analytical performance to similar variants that are companion diagnostics?

- In your opinion, would evidence of a clinical trial (NCT number) be sufficient to include the variant on the panel? Should this be gene or variant based clinical validity?

Claim Modifications

Consider a variant with demonstrated analytical validity in lung tissue (already on the panel and listed in table 2 of the intended use), and a new companion diagnostic claim in colon cancer. What level of validation should be needed to move a variant to Table 1 of the intended use when new targeted therapeutics are approved?

- Does it need analytical validation in the tissue of interest?

- What about new variants that were not previously reported in Table 2?

Labeling of an NGS-based Oncology Panel

We are interested in how to truthfully and accurately provide any necessary disclaimers in the labeling of these panels.

- What are the disclaimers that should be included in labeling around issues of panel comprehensiveness? Please consider cases of absence or inadequate coverage of genes/variants with associated therapeutics or disease states, absence or inadequate coverage of known hotspots, exon, and other variations in panel composition that could impact assay interpretation.

- What disclaimers should be included for variants reported by the assay but not included in Tables 1 or 2?

- What disclaimers should be included for de novo variant reporting as opposed to pre-defined variant reporting?

New Assay Performance Characteristics

One concern with follow-on companion diagnostic claims is that the new assay may have different/better analytical sensitivity than the original assay.

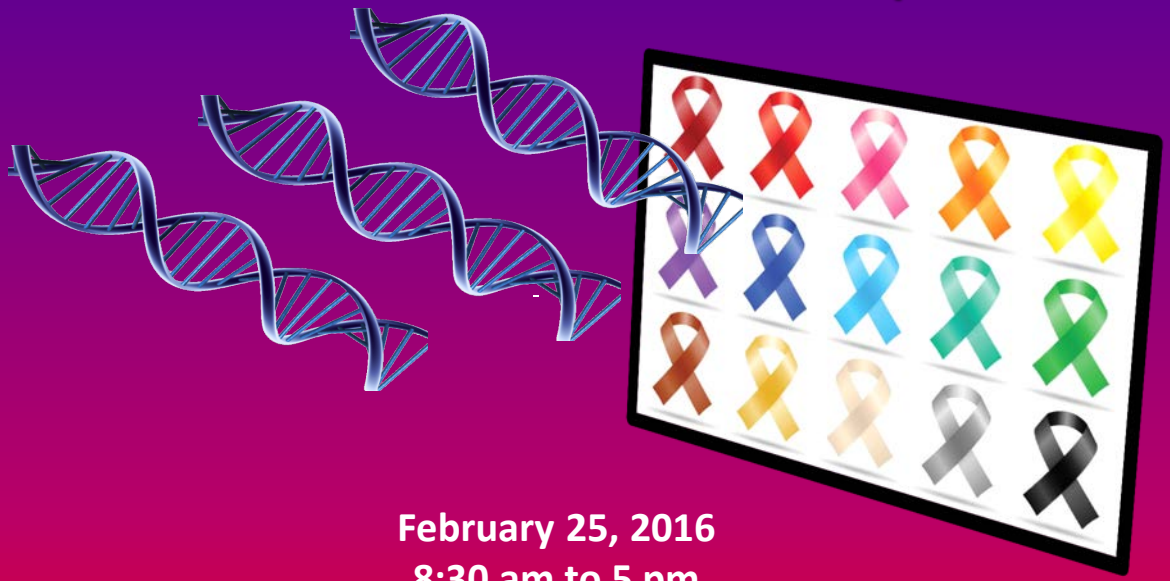
- How concerned are you about the clinical meaning of improved sensitivity?
- What if the patient population changes because of increased sensitivity?
- Imagine an assay with 100x greater sensitivity that would identify a significantly different patient population. How do you ensure that clinical benefit of the drug to this population would be assured?

BREAK

3:00-3:30 pm

FOOD AND DRUG ADMINISTRATION

Next Generation Sequencing-based Oncology Panels Public Workshop



February 25, 2016
8:30 am to 5 pm

White Oak, MD

Webcast address: https://collaboration.fda.gov/ngsop0216/

FDA's Medical Devices News & Events Workshops & Conferences calendar:
http://www.fda.gov/MedicalDevices/NewsEvents/WorkshopsConferences/default.htm

Open Public Comment

Moderators: Anand Pathak, M.D., Ph.D.
and Soma Ghosh, Ph.D.

Each presenter has been allotted 4 minutes.

Public Comment Speaker #1

Dara Aisner, M.D., Ph.D.
University of Colorado

ASSURING QUALITY IN ONCOLOGY NEXT GENERATION SEQUENCING

DARA L. AISNER, MD, PHD

UNIVERSITY OF COLORADO

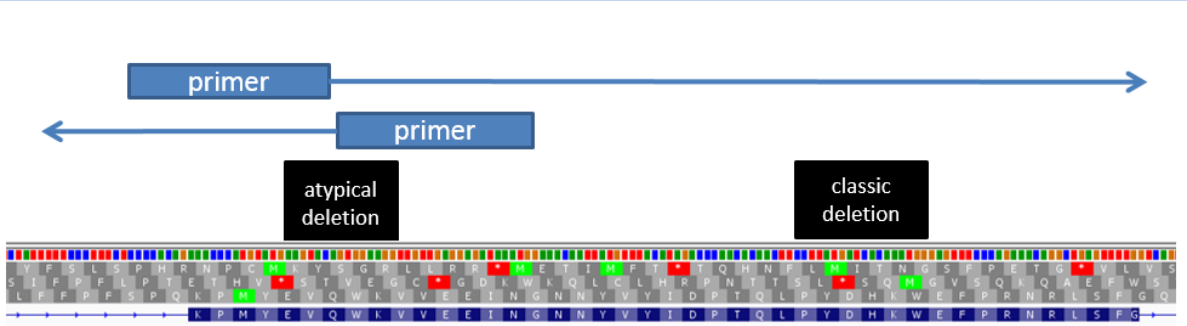
DISCLOSURES:

HONORARIA: ASTRAZENECA, CLOVIS ONCOLOGY

CONSULTING: CASDIN CAPITAL, OXFORD ONCOLOGY

ASSAY VALIDATION

- Validation
  - Key concern: No depth of available specimen bank, nor any designed reference material can cover every possible variant type
    - Locations of probes/primers can vary, which will impact the detected alterations



KIT exon 11

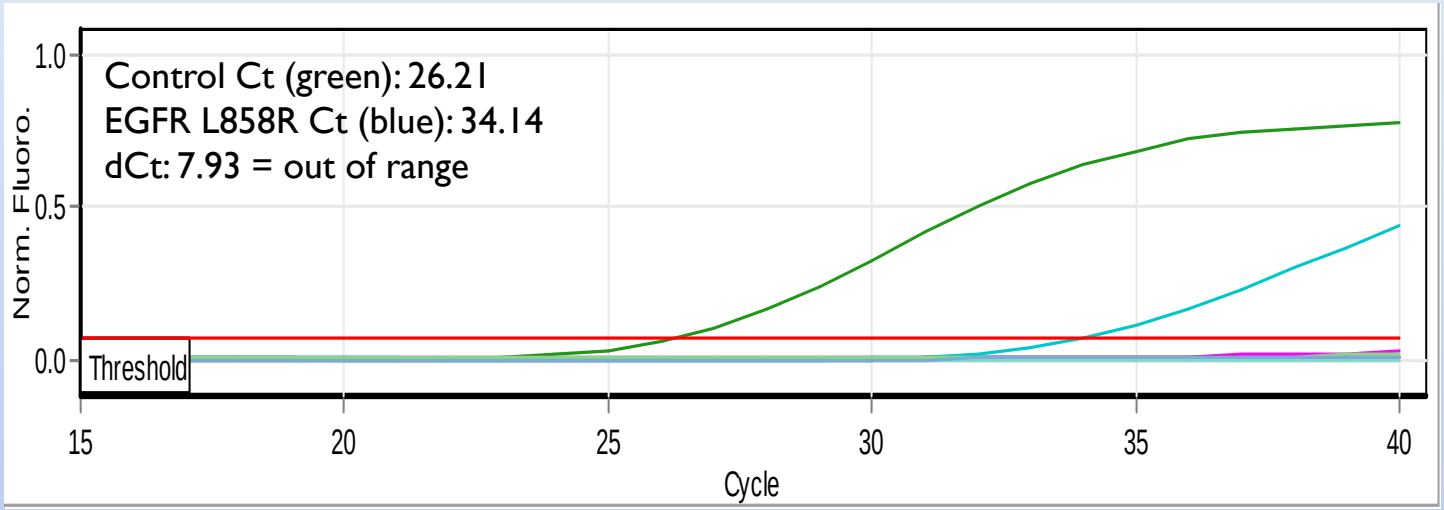
ONGOING QUALITY CONTROL

- **Ongoing quality control**
  - **Use of appropriate QC tools (controls, metrics etc)**
  - **Internal laboratory quality control approaches (repeat, orthogonal etc)**
  - **Proficiency testing**
    - **You don't know what you don't know**

FLEXIBILITY

- Flexibility
  - The ability to identify and then accommodate to identified weaknesses always serves to improve the quality of testing
    - ‘Lock in’ prevents such adaptation

ABILITY TO SEE THE DATA



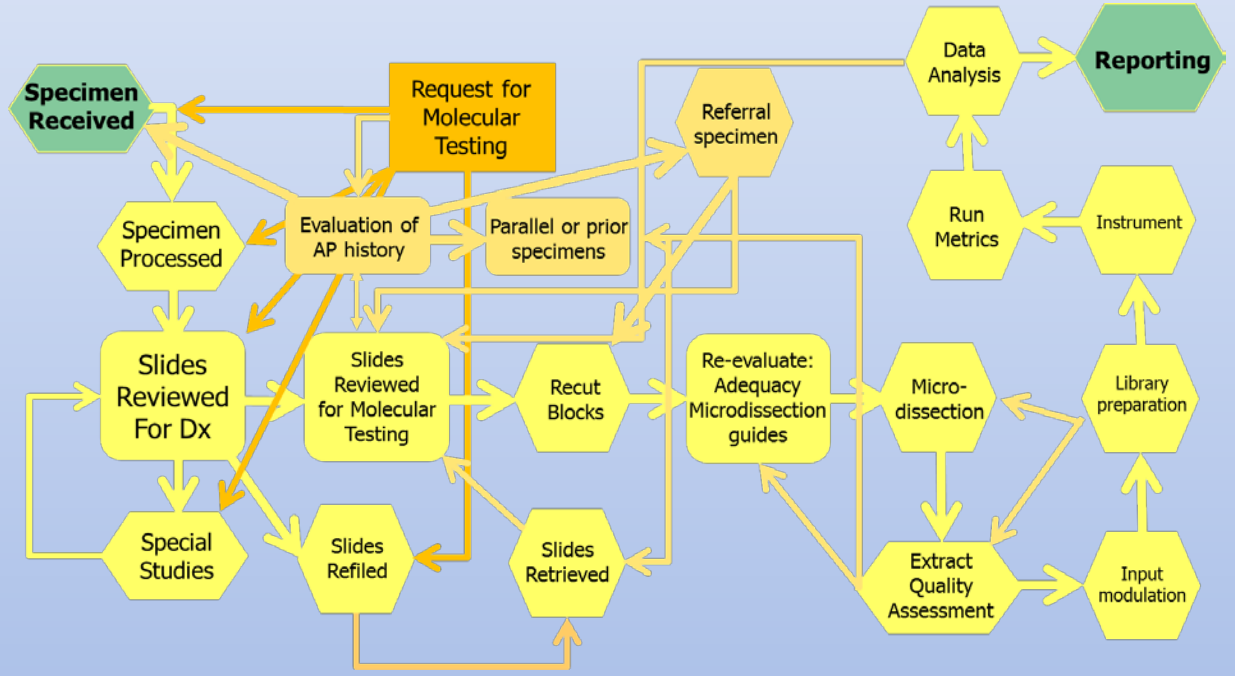
Low tumor cellularity sample

If run in FDA-compliant mode, this would have been resulted as 'negative'

Repeat sampling showed strong, unequivocal *EGFR* mutation

PROFESSIONAL INVOLVEMENT

- No such thing as sample in – result out when there is this much complexity



≠

Wisegeek.net

Public Comment Speaker #2

Tobias Guennel, Ph.D.
Precision for Medicine

PRECISION FOR MEDICINE℠

THE FUTURE OF MEDICINE IS PRECISION

FDA Workshop on NGS Oncology Panels: Public Comment

Tobias Guennel, PhD

Director of Analytics, Precision for Medicine

February 25, 2016

Focus of Comments

Analytical Validation and Bioinformatics:

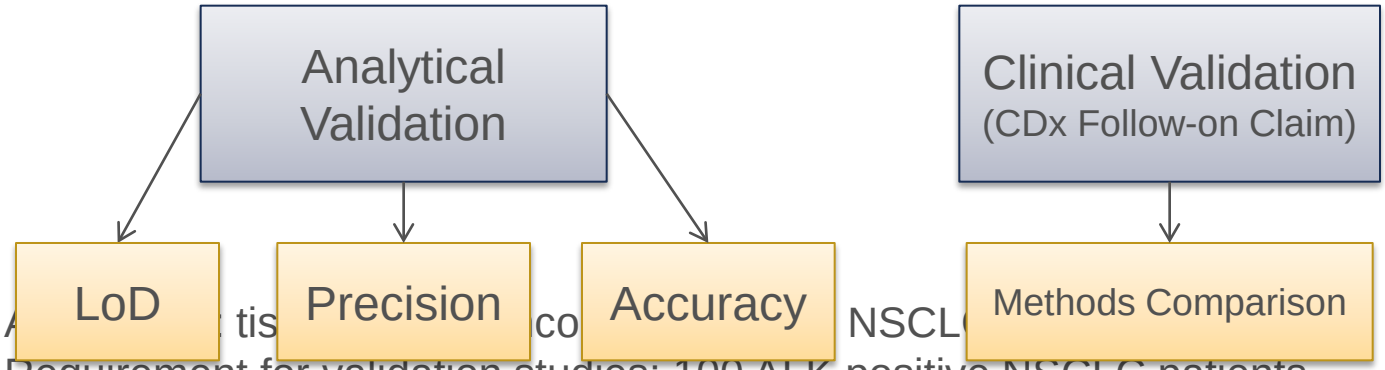
- *Q7: Should commutability studies be conducted in order to infer the performance of the assay on clinical samples from data obtained in cell lines or plasmids?*
- *Q11: Are there risk-based strategies can be employed by FDA and manufacturers to determine when bioinformatics pipeline changes have significant potential to impact assay performance?*

Clinical and Follow-on Companion Diagnostic Claims:

- *Q1: Are there key considerations for evidence that would or would not be sufficient for providing a reasonable assurance of safety and effectiveness for a follow-on companion diagnostic claim?*
- *Q4: Is there a specific level of clinical evidence that should be provided in order to move a variant from Table 2 to Table 1 of the proposed general intended use above when new targeted therapeutics are approved?*

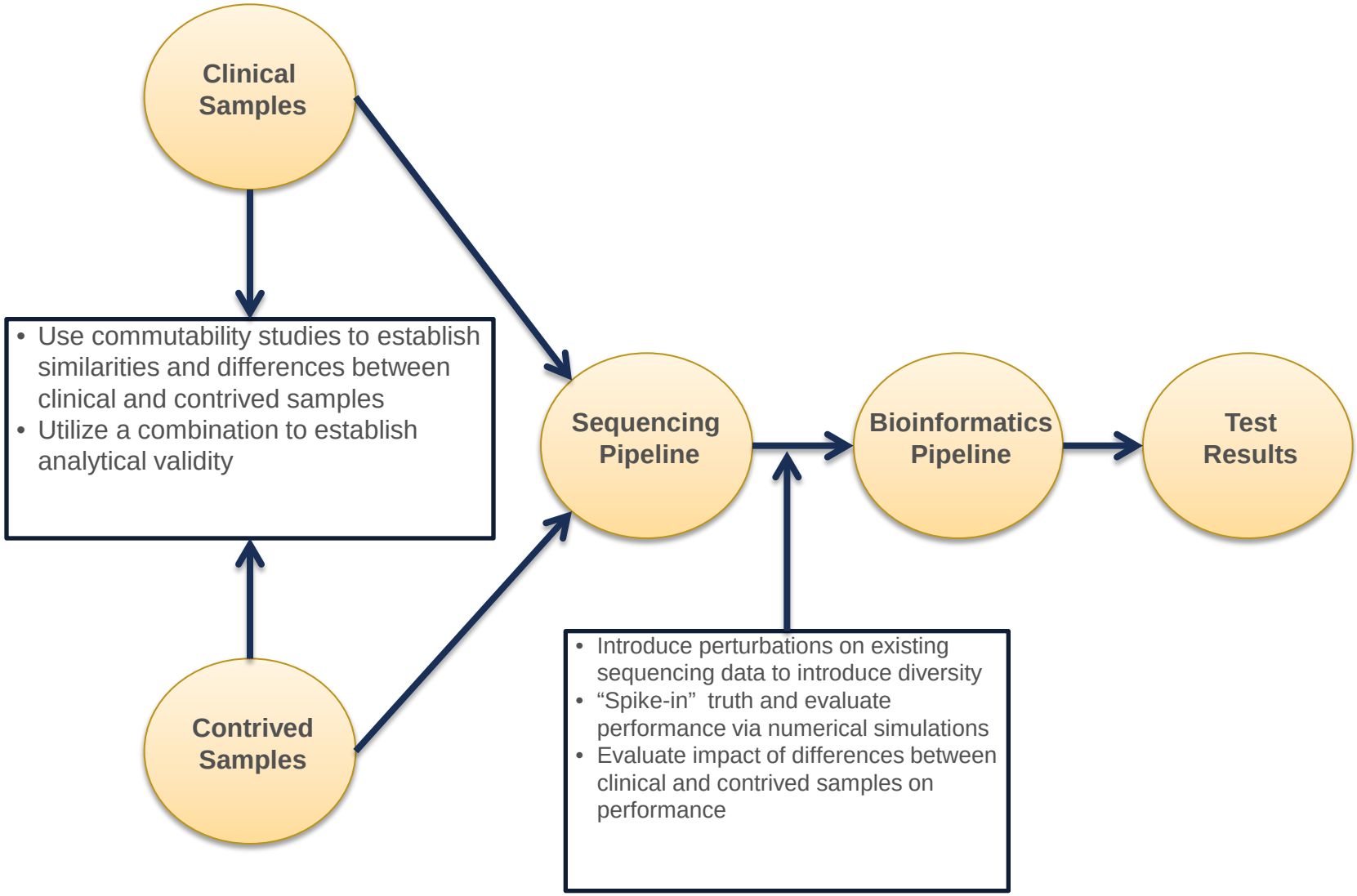
Sample Size Considerations Using Traditional Approach

- Studies that traditionally involve clinical specimens:

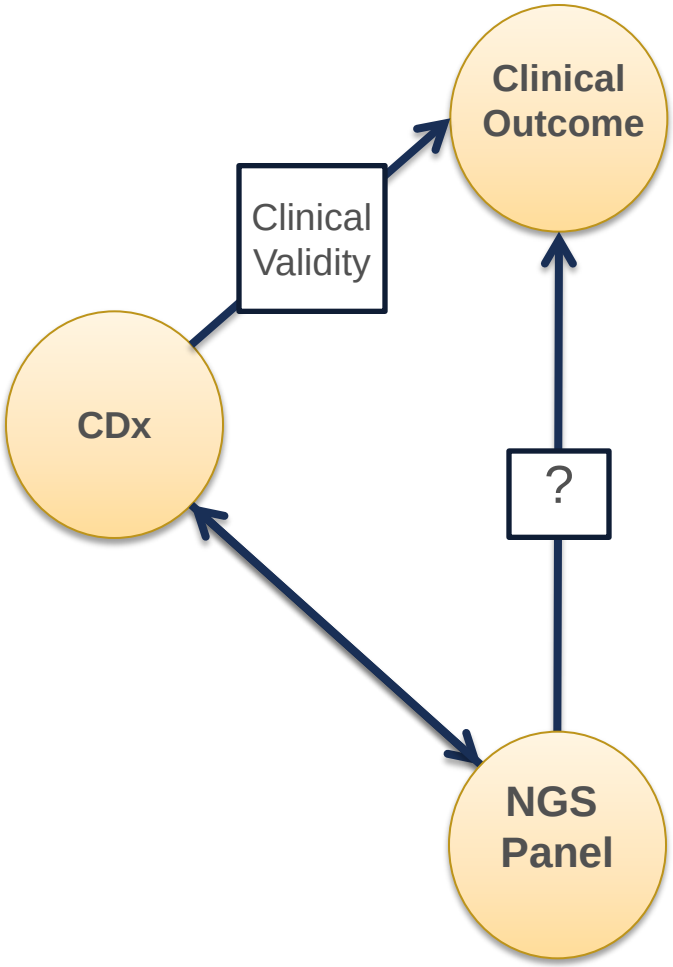


- A tis co NSCL
- Requirement for validation studies: 100 ALK positive NSCLC patients
- ALK prevalence 1%: >1000 NSCLC patients need to be screened
- Complicating factors:
  - Sufficient tissue to run both test of interest and CDx potentially across multiple studies must be available
  - Intended use population may be restricted to Stage III / IV where biopsies are difficult to obtain
  - Reference methods with sufficient sensitivity may not be available

Potential Approaches for Analytical Validation



Potential Approaches for Clinical Validation



- Can statistical approaches be leveraged to evaluate clinical validity and supplement clinical validation studies?
  => Simulation studies may be a viable approach to evaluate impact of different parameters on performance
  (e.g. concordance between CDx and NGS Panel)
- Are adaptive designs a viable alternative to establish clinical validity in a phased approach to alleviate burden of large number of clinical samples in a short amount of time?
- Can simulation studies be utilized to evaluate impact of using contrived samples on establishment of clinical validity?

Take Away Points

- Potentially 1000s of patients will need to be screened just for one submission
  => Very challenging for NGS oncology panels that potentially evaluate multiple variants with low prevalence across multiple indications
- Alternative approaches to the traditional testing paradigm are needed
- Questions in discussion paper show the progress that has been made in identifying challenges and potential angles to identify solutions
- Richness of data that are generated by NGS panels can be leveraged to evaluate analytical validity (and potentially clinical validity)
  - Example: evaluate performance across representative variant categories
- In-silico approaches may be a viable supplemental approach to evaluate impact of parameters on performance for both analytical and clinical validity

Public Comment Speaker #3

James Willey, M.D.
University of Toledo

Strategies to establish performance characteristics for NGS-based rare variant oncology panels

FDA Workshop February 25, 2016

James Willey, MD
Co-Founder and Consultant, Accugenomics, Inc.
George Isaac Chair for Cancer Research
University of Toledo Health Sciences Campus

Tom Morrison, Ph.D.
Chief Technology Officer, Accugenomics, Inc. Wilmington, NC, USA

Determining Confidence for Each Rare Variant Fraction Measurement

H23:H520 Cell Line DNA Mixture

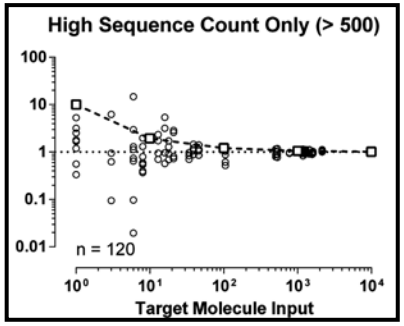
Synthetic Competitive Internal Standards (IS)

Library preparation (target enrichment)

Illumina Hiseq 2500 Sequencing Platform

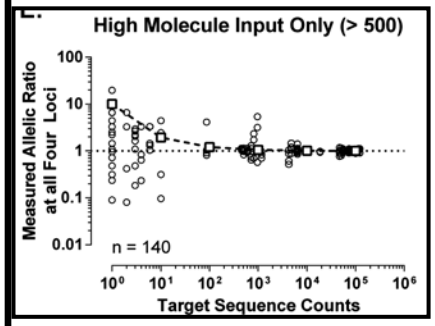
Analysis Pipeline

IS Control for sampling error:
Library prep: Low amplifiable target copies loaded (e.g., FFPE, cytologic)



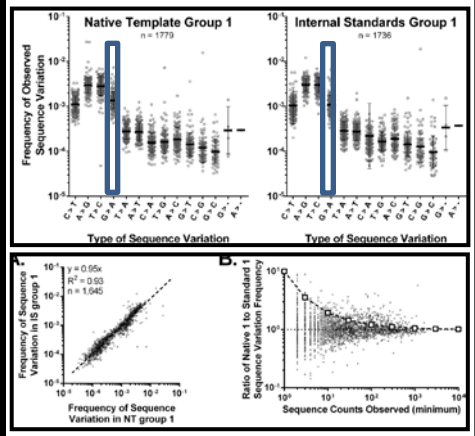
Symbols:
H23/H520 rs735482 allelic ratio

IS Control for sampling error:
Sequencer: Inadequate sequencing space for samples/targets (e.g., excessive/unequal loading)



H23/H520 1:1 library serially diluted
Symbols: rs735482 allelic ratio

IS Control for polymerase/sequencing error:
Error in IS and target statistically the same



Inter-nucleotide and inter-regional variation in sequence error rate

Determine confidence for each value:

- **Estimated sampling error based on:**
  - known amplifiable copies loaded into library
  - Amplicons loaded into sequencer
- **Estimated sequencing error:**
  - Infer from known error rate in IS

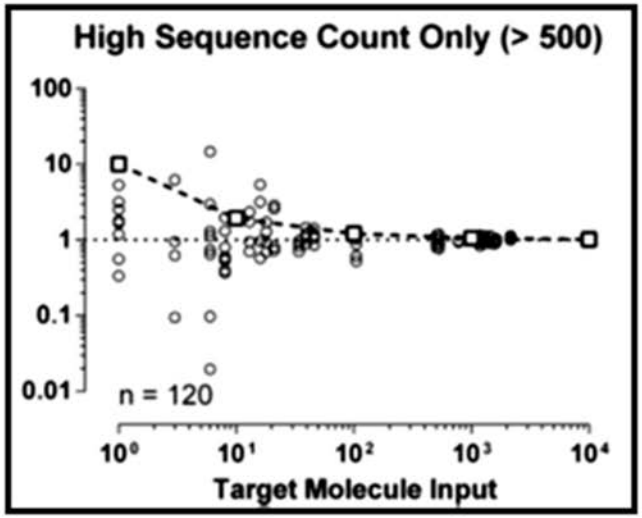
Inadequate loading at each step independently increases measurement imprecision

Biomol Detect Quantif. 2015 Sep 1;5:30-37

Determining Confidence for Each Rare Variant Fraction Measurement

IS Control for sampling error:

Library prep: Low amplifiable target copies loaded (e.g., FFPE, cytologic)



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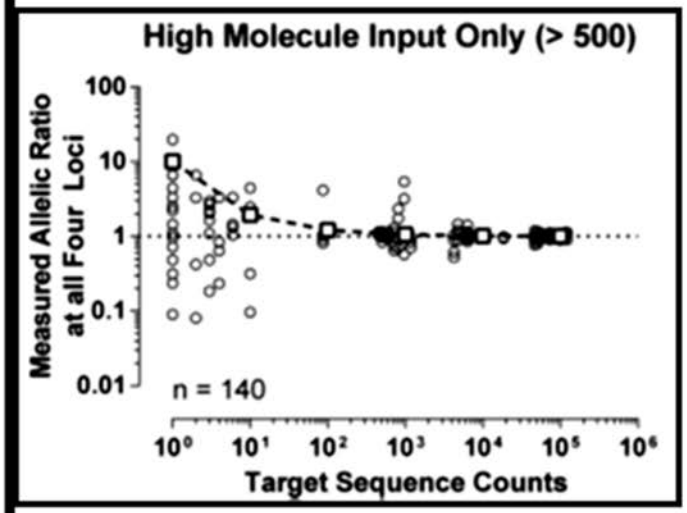

H23/H520 1:1 library serially diluted
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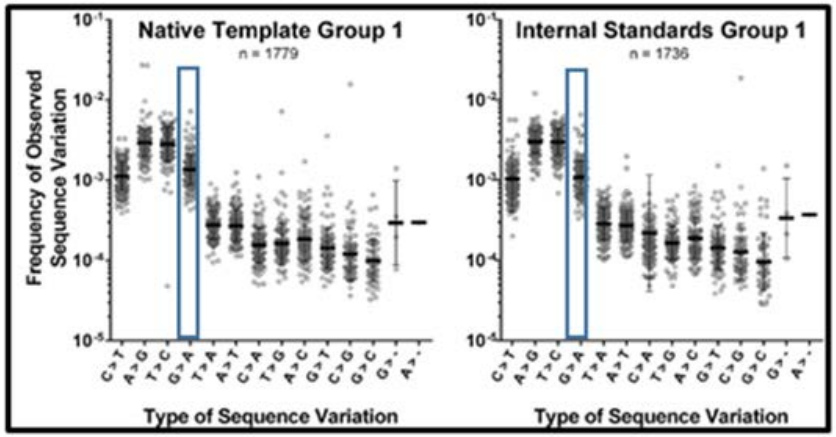
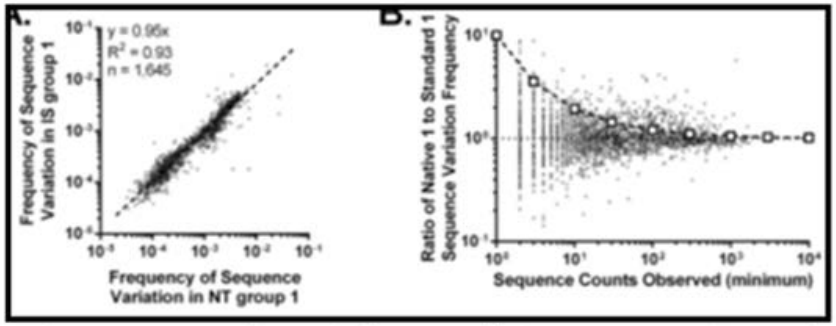
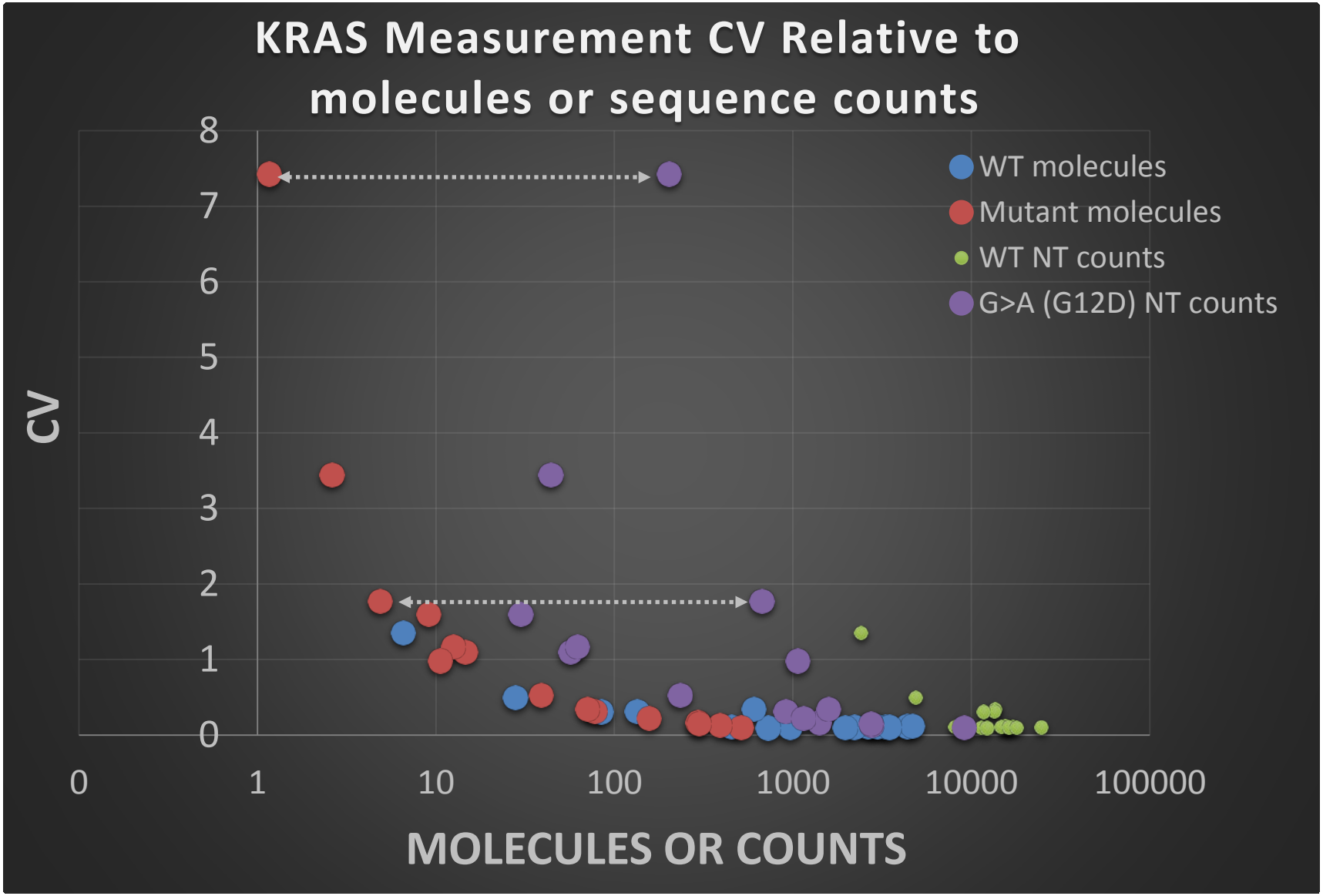
Determining Confidence for Each Rare Variant Fraction Measurement

IS Control for polymerase/sequencing error:
Error in IS and target statistically the same





Inter-nucleotide and inter-regional variation in sequence error rate



Key Point: Sequencing Depth alone is not sufficient quality control criterion

Conclusions Regarding Analytical Performance:

- CV should be estimated for each variant fraction measurement value based on
  - Molecules loaded into library
  - Library amplicons measured in sequencer
- Synthetic IS in each measurement as process controls is an efficient way to estimate CV for each value and sequencing error at each nucleotide.
- Any departure from optimal conditions will be associated with higher LOD.
- Sub-optimal conditions are frequent, unpredictable, and can render 5% measurement unreliable
  - For example, quality and size of sample, reagents, library preparation.

Conclusions:

- **Under optimal conditions (i.e., 50,000 amplifiable copies loaded into library, 1,000 library amplicons sequenced)**
  - **Limit of quantification (LOD) for KRAS G12D mutation fraction on Illumina Hiseq 2500 will be > 0.004 (>0.4%) assuming:**
    - **200 mutated copies, 50,000 WT copies, 1,000 sequences measured for each value.**
      - **This will be associated with CV = 20%**
    - **0.2% sequencing error on Illumina Hiseq 2500 at KRAS G12D site.**
    - **LOD defined as 3σ above background (sequencing error)***

Public Comment Speaker #4

John Sninsky, Ph.D.
CareDx

FDA Public Workshop on Next Generation Sequencing-Based Oncology Panels

John J. Sninsky, PhD
Chief Scientific Officer
CareDx, Inc.
Brisbane, CA

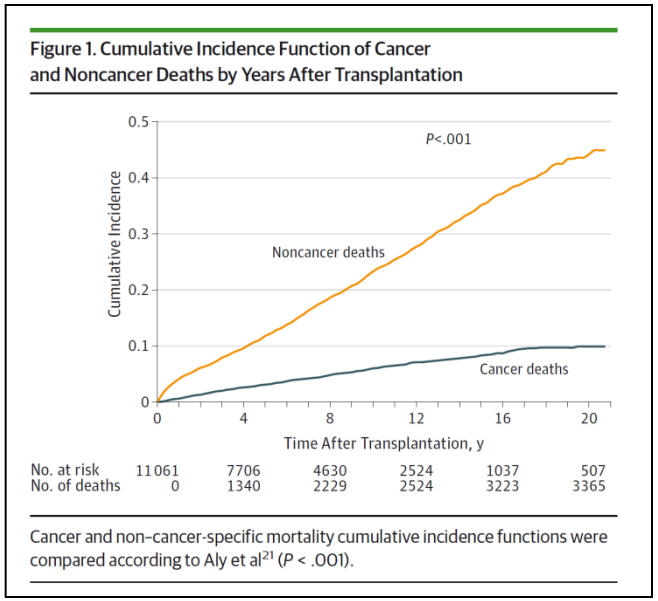
February 25, 2016

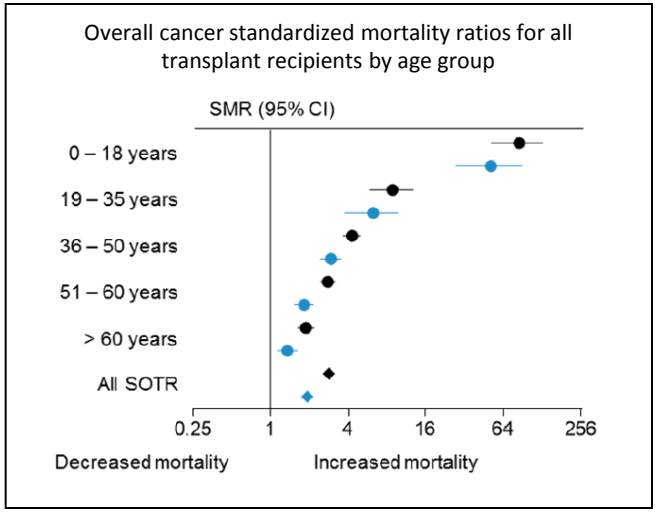
Cancer in Organ Transplant Setting

- Organ transplant patients are at **significantly elevated de novo cancer risk** due to requisite chronic immunosuppressive therapy
  - Younger organ transplant patients are at even higher risk than older patients

- Organ transplant patients who develop cancer have been reported to **experience worse outcomes** than patients with cancer in general population

- CareDx is a molecular diagnostics company focused on the discovery, development and commercialization of clinically differentiated, **high-value, personalized diagnostic surveillance solutions for transplant recipients**





Acuna *et al. JAMA* Oncol Epub ahead of print (2016).
Engels *et al. JAMA* 306, 1891 (2011).

Fit-for-Purpose Design and Individual Performance Standards are Important

- NGS-based cancer panels produce data for numerous variants and it is not always feasible to design, develop and review the performance of all potential individual variants in the test

  - Design concept standards
    - Select **representative variants** with **boundary properties** from different regions to reflect reasonable demonstration of device's overall performance
    - Performance metrics for **some variants may still not be inferred** with high confidence
  - Individual performance standards
    - Development of **individual performance metrics for each variant is burdensome**
    - Due to technological challenges of some sequencing variants, assurance of **inferred performance may not be suitable**
  - **Qualitative or quantitative tests** depending on indication

Clinical-Grade and Research-Grade NGS are Different

	Research-Grade	Clinical-Grade	Comments
Reference materials	Internal specimens / External specimens	**External standards; orthogonal technology validation**	Ensures high test accuracy (e.g. Horizon Discovery)
Methods-based proficiency	Rarely used	**Performed regularly**	Ensures high test reproducibility (e.g. NIST-GIAB reference genome)
Information tracking systems	Sometimes used	**Always use LIMS**; some integration with EMRs	Ensures sample and reagent tracking ; correct report for each patient sample (e.g. StarLIMS, GenoLogics)
Bioinformatic analysis	Open source combined with subscription/license; frequently changing; & early adoption of new software/algorithms	Open source combined with subscription /license; use mature software and CDS **Locked, change controlled, requires re-validation**	Ensures test consistency and reproducibility (e.g. DNAnexus – platform also selected by FDA as part of PrecisionFDA initiative)
Validation of steps in process	Sometimes	**Always**	Follow applicable CLIA-CAP NGS recommendations/guidelines to ensure highest quality of the test
Variant Content	Mixed sources	**High data quality, high confidence database operations as well as timely and sourced interpretive evidence**	Rules-based decision support to capture drug and diagnostic test labels and guidelines to aid interpretation (e.g. ClinVar, COSMIC, Qiagen (Ingenuity), LOVD, Mycancergenome, HGMD, etc.)

Horizon Reference Materials Permit Performance Evaluation of Cancer Panel Tests

Horizon Reference Material	Chromosome	Gene	Variant	Expected Allelic Frequency	Qiagen Clinically relevant panel (101x)[1]	Qiagen Actionable mutations panel (201x)[1]	Illumina TruSight Tumor 15
5% Multiplex Reference Standard (HD777)	7p12	EGFR	L858R	5.0%	5.3%	Below 5% [1]	5.1%
	7p12	EGFR	T790M	5.0%	6.3%	6%	5.0%
	12p12.1	KRAS	G12D	6.3%	6.5%	5.8%	6.7%
	1p13.2	NRAS	Q61K	6.3%	7%	6.7%	5.9%
	1p13.2	NRAS	A59T	6.3%	6.6%	6.6%	7.1%
	3q26.3	PIK3CA	E545K	6.3%	6.8%	Not in panel	7.5%
5% BRAF V600E Reference Standard (HD773)	7q34	BRAF	V600E	5%	5.9%	5.5%	3.7%

[1] 5% filter used for this analysis

Methods-based Proficiency is Critical

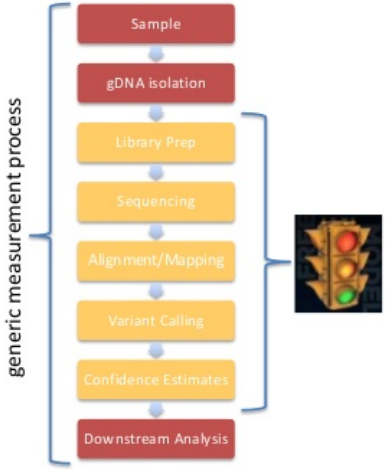
NIST Human Genome Reference Materials (RMs)

- NIST RM 8398 is available!
  - tinyurl.com/giabpilot
  - DNA isolated from large growth cell cultures
  - Stable, homogeneous
  - Best for regulated uses
  - DNA from same cell line at Coriell (NA12878)

Genome in a Bottle Consortium genomeinabottle.org

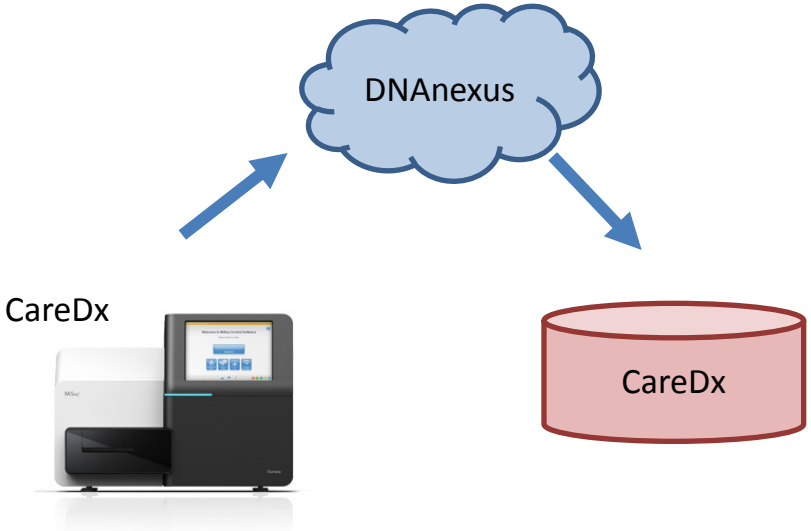
Measurement Process

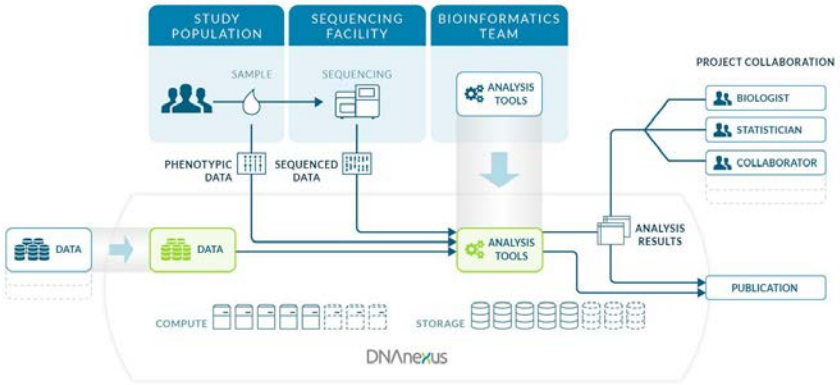
- gDNA reference materials will be developed to characterize performance of a part of process
  - materials will be certified for their variants against a reference sequence, with confidence estimates



- Alternative assessment procedures complement analyte-specific proficiency
- Delineates a step or process within the entire workflow for review
- Develop multiple reference genomes

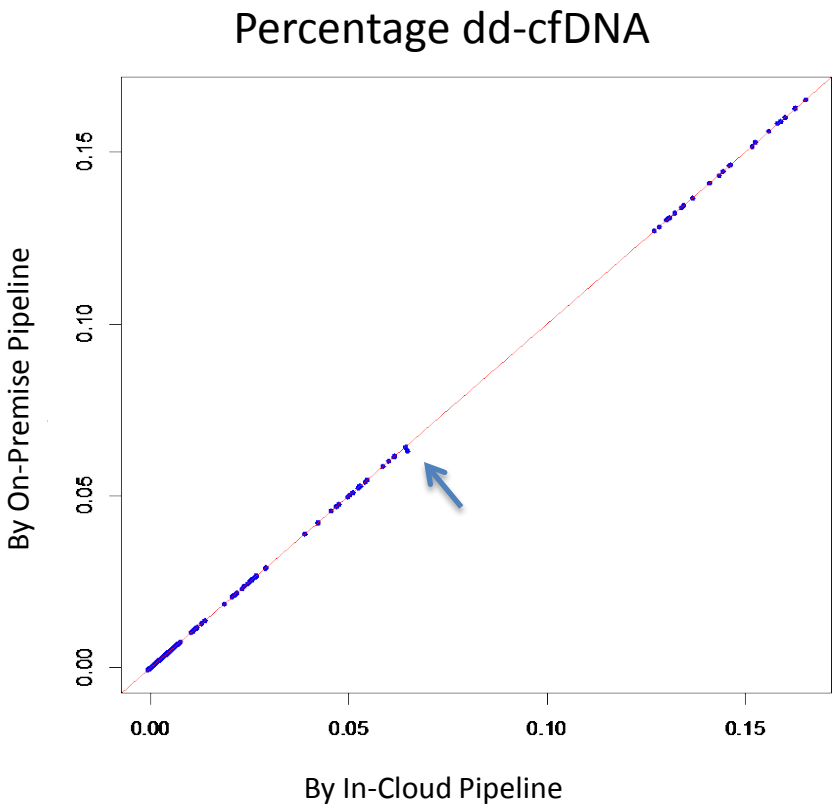
Standardization of Computational Analysis is Paramount

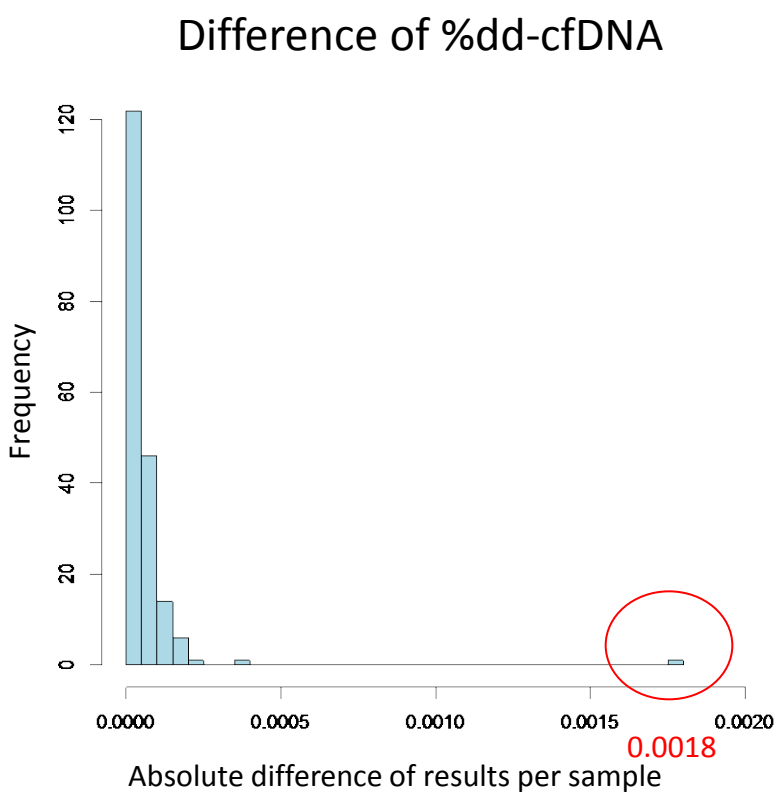




- Explore and encourage cloud based computing to permit independent review of custom pipeline analysis
- Use best-in-class software modules
- *In-silico* constructed standards can play an important role in computational validation

Essential to Demonstrate Concordance Between On-Premise and Cloud Pipelines





Ratios of donor-derived cfDNA computed by the pipeline in DNAnexus Cloud and by pipeline on CareDx local cluster are essentially identical. One sample has slightly greater difference in results (0.0018%) from the two pipelines. This difference was tracked to a slight difference between the bcl2fastq versions running on the MiSeq and the standalone version.

Summary

For clinical-grade NGS CDx cancer panel testing, my colleagues at CareDx and I encourage the FDA and our industry to

- Use **clinical-grade sequencing** procedures
- Consider **fit-for-purpose criteria** for different cancer tests (both qualitative and quantitative tests)
- Develop and utilize **well-characterized, sustainable reference materials** to evaluate cancer test panels
- Use pre-existing recommendations for **methods-based proficiency** testing in conjunction with the availability of exemplary genome reference resources such as Genome in a Bottle and NIST
- Iteratively **review high confidence regions** and **improve lower confidence regions** of NIST reference genome
- Implement rigorous and **standardized strategies** for computational pipeline analysis – not “black boxes”
- Identify **flexible and adaptable regulatory approaches** to address dynamic accumulation of evidence

Public Comment Speaker #5

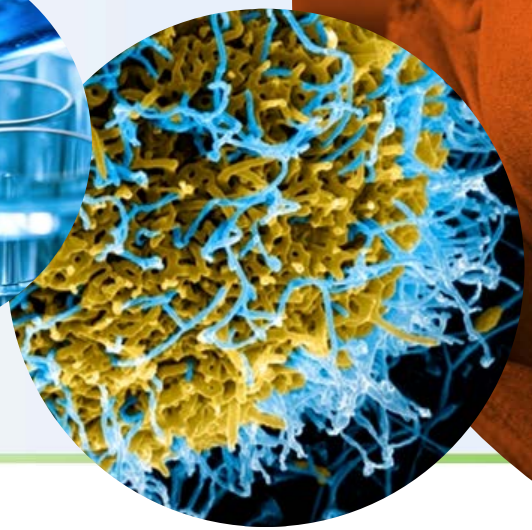
Roger Klein, M.D.
Association for Molecular Pathology

Public Comment Speaker #6

Garlick Russell, Ph.D.
SeraCare Life Sciences

Highly Multiplexed Controls for NGS Assays

Russell Garlick PhD
Chief Scientific Officer
rgarlick@seracare.com



Acknowledgements to Interlab Group: NCI MoCha, Dartmouth Hitchcock, Weill Cornell, Virginia Commonwealth, Bio-Reference Labs, Jackson Labs, SCLS, Beta Innovations

Analytical Validation and QC

1. Pre-analytical validation and QC
   a) Extracted DNA / RNA from patient sample is the internal control
2. Sequencing and pipeline validation and QC
   a) Highly multiplexed assays require highly multiplexed reference materials
   b) Pool results to increase sample size and apply appropriate statistics
   c) Precise %AF required to trend data, challenge LOD
   d) Best way to know if you can detect a variant is to test it

Interlab Study

Materials (52 detectable variants per run)

- 26 X 1000-bp biosynthetics including 4 SNVs in homopolymer regions, 4 INDELS and 18 SNVs into a HapMap gDNA background GM24385 at two allelic frequencies. Each plasmid includes an actionable variant and a unique 6-bp internal quality marker *

Methods

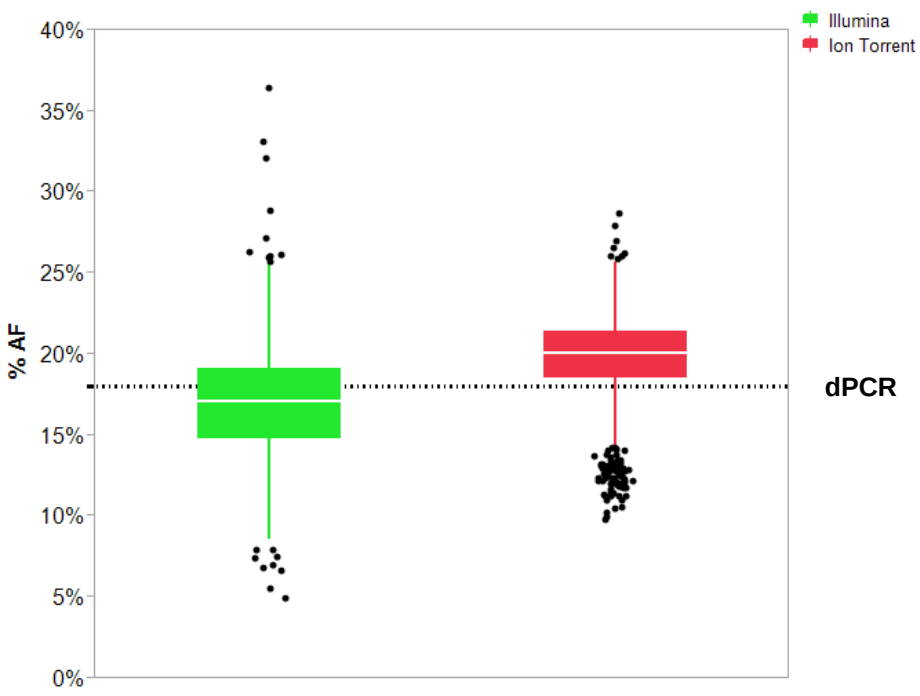
- dPCR is used as the orthogonal method to measure %AF
- Tested at 6 CLIA certified labs and 1 research lab, 119 runs for a total of 357 assays over 8 weeks

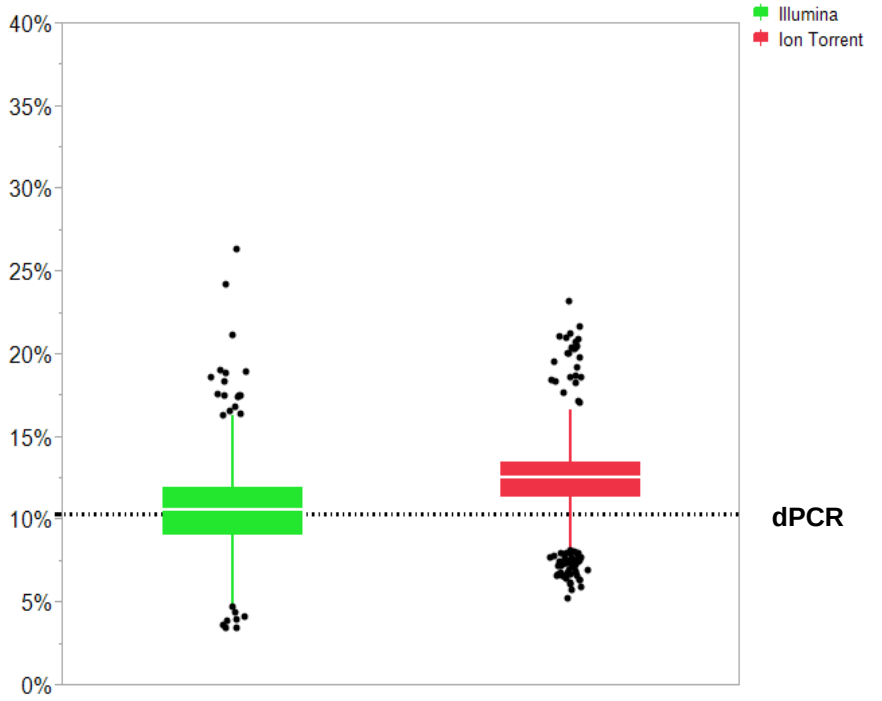
On-going analysis, preliminary data shown

- Non-parametric analysis for comparisons
- Outliers for pass / fail using binomial distribution

* NCI MoCha CRADA Mickey Williams, Jason Lih et al

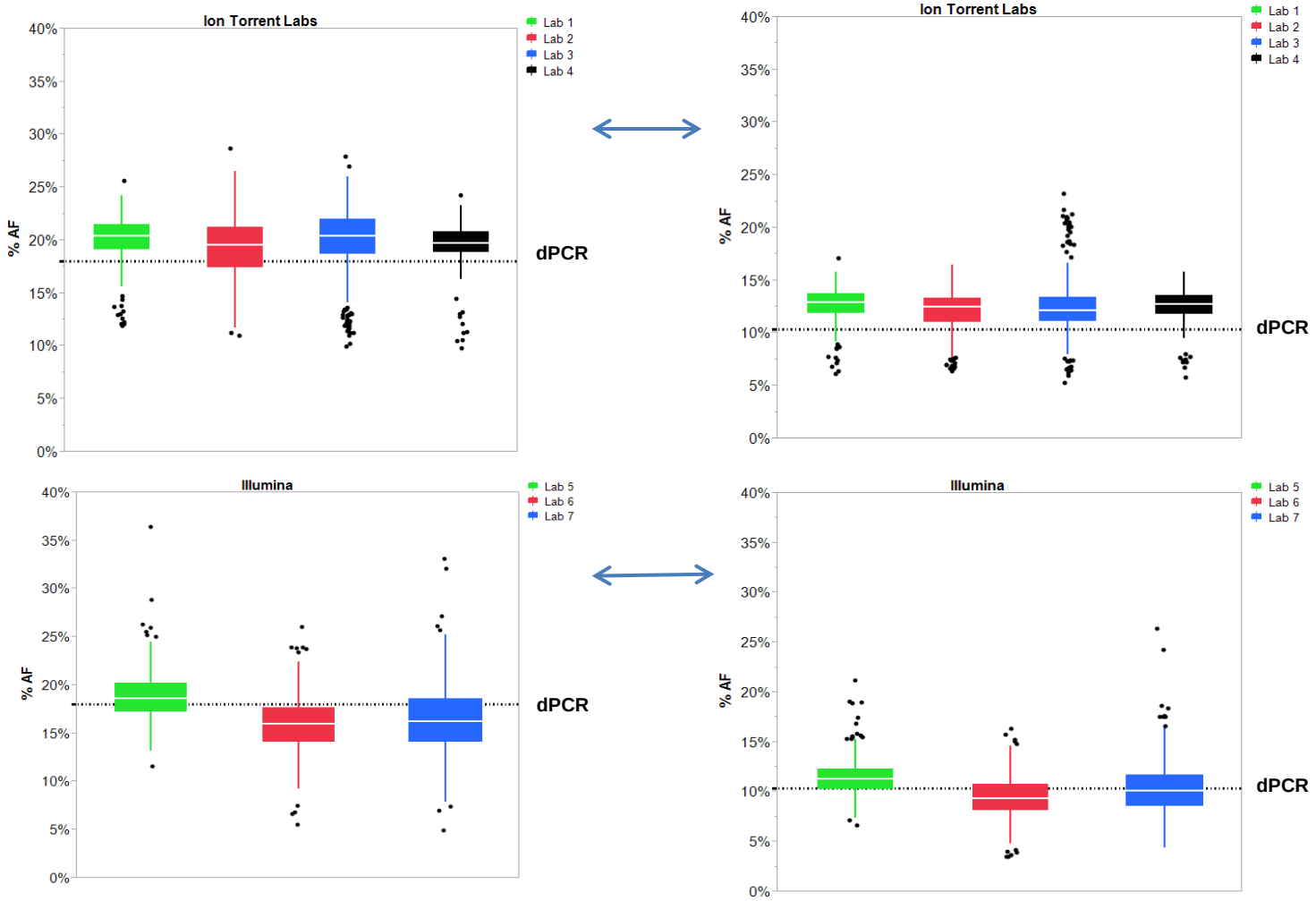
Compare Platforms



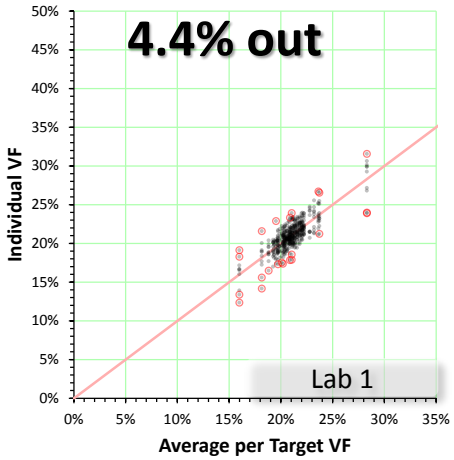


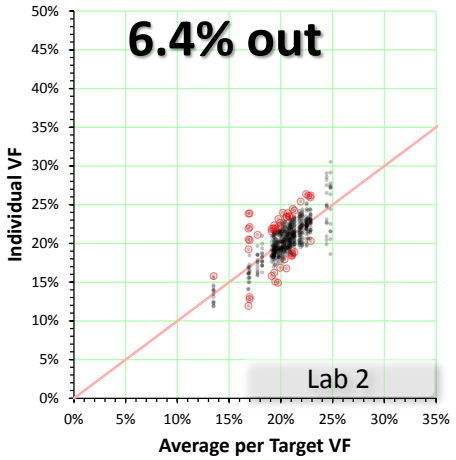
- Also compare other platforms, labs, types of variants detected, formats, LODs and pipelines

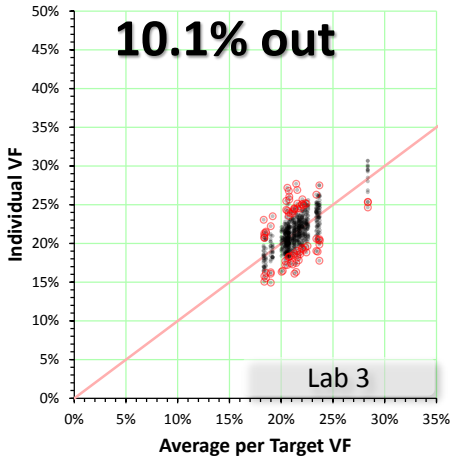
Interlab, Same Platform Comparison

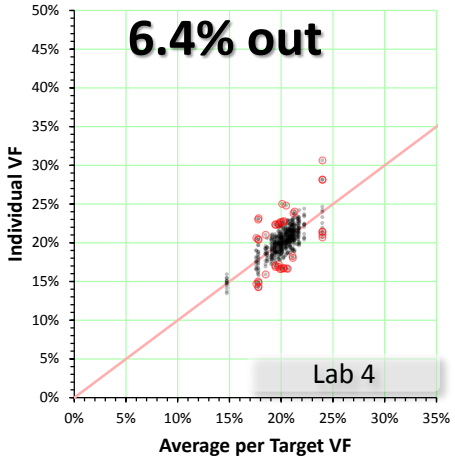


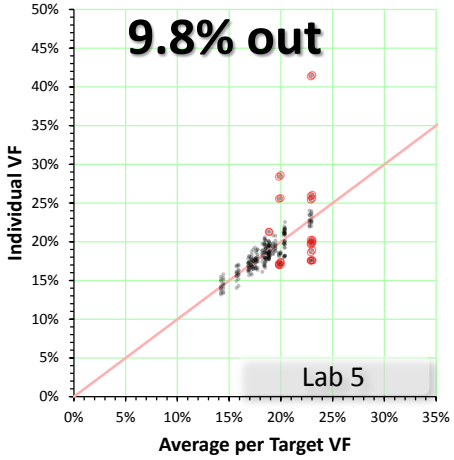
Outlier Analysis Using 95% Binomial Prediction

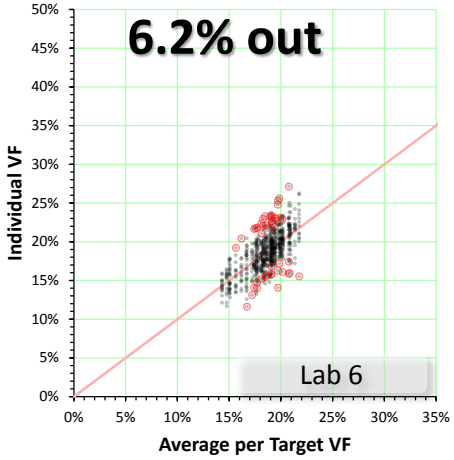


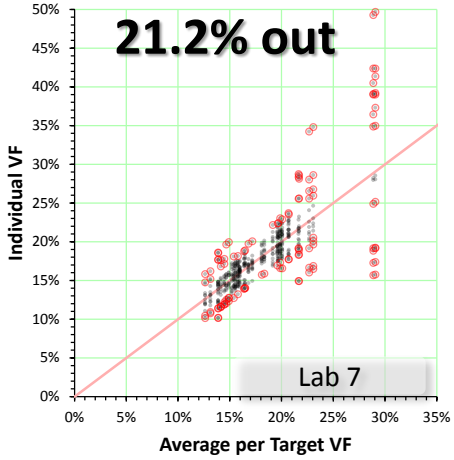












- Individual %AF (y-axis) vs historical average %AF (x-axis)

Recommendations for Analytical Sequencing Validation and Bioinformatics

- Use highly multiplexed (>50 variants) controls and reference materials for validation and QC
  - allows pooling of data and a greater ability to compare performance
  - greater chance to detect true assay variability with larger data sets
- Use outlier testing by binomial distribution to complement other trending reports, Levey-Jennings, as important metrics to accept or reject an NGS run
- Use dPCR as an orthogonal quantitation method
- Controls and calibrators should have a flexible design, easy to add new variants, include all types at different %AF

Thank You

Contact Information

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Public Comment Speaker #7

Natalie LaFranzo, Ph.D.
Horizon Discovery

HORIZON DISCOVERY

Monitoring NGS Oncology Panel Performance using Cell-line based Reference Standards

FDA Workshop - February 25, 2016

Natalie LaFranzo, PhD
Product Manager – NGS Products

horizon

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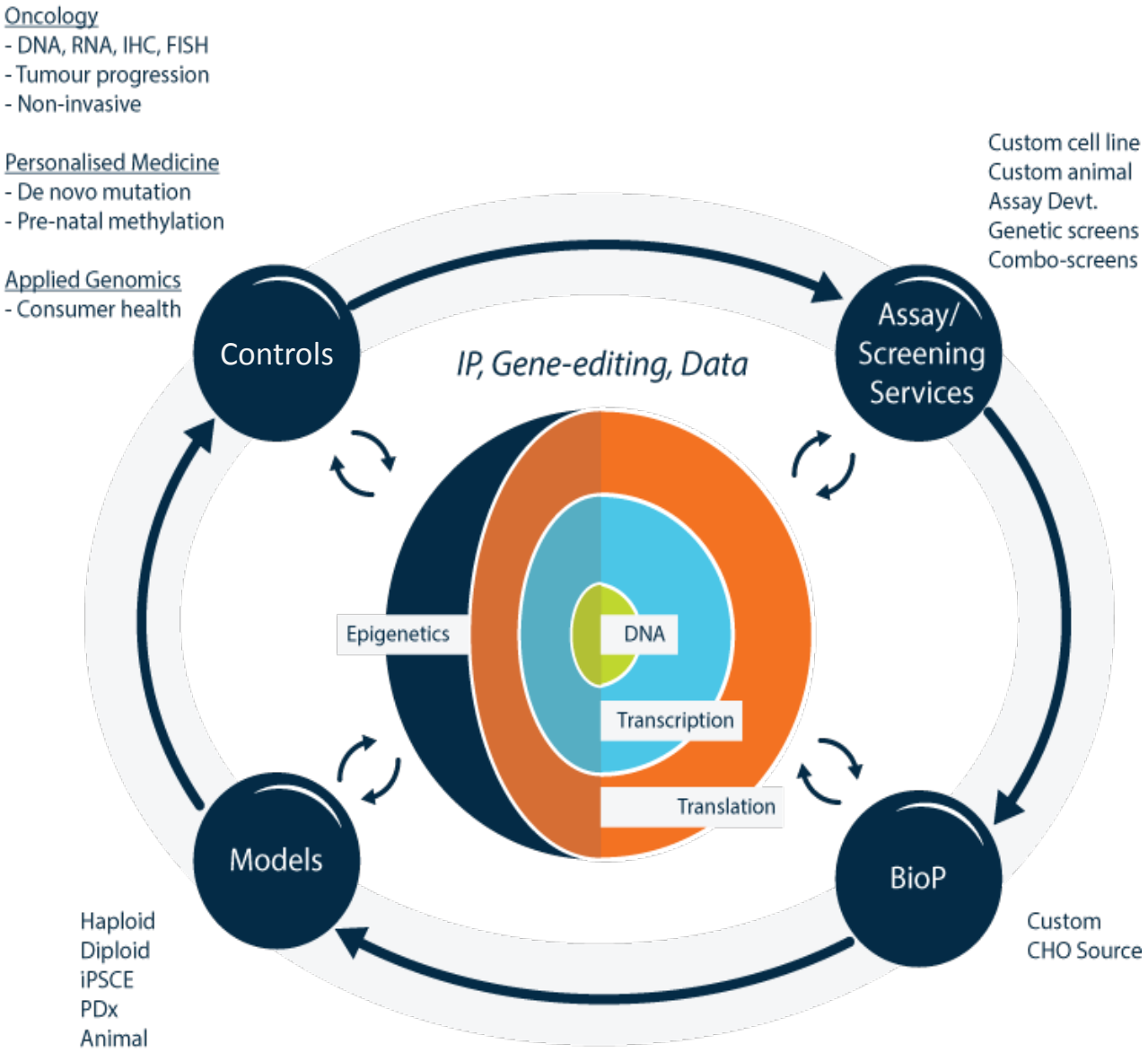
About Horizon Discovery – Cell Line Builders



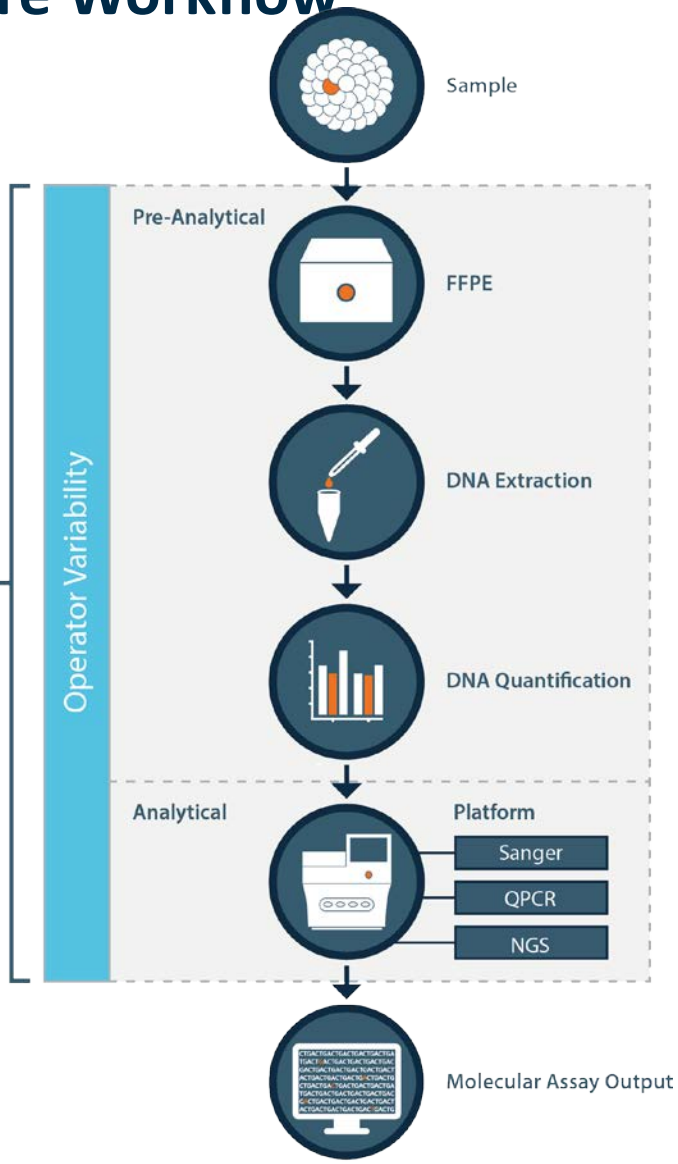
Quality Control – Throughout the Entire Workflow

HDx Reference Standards offer a sustainable source of reference material to laboratories, proficiency schemes and manufacturers, providing an unprecedented level of control.

horizon diagnostics

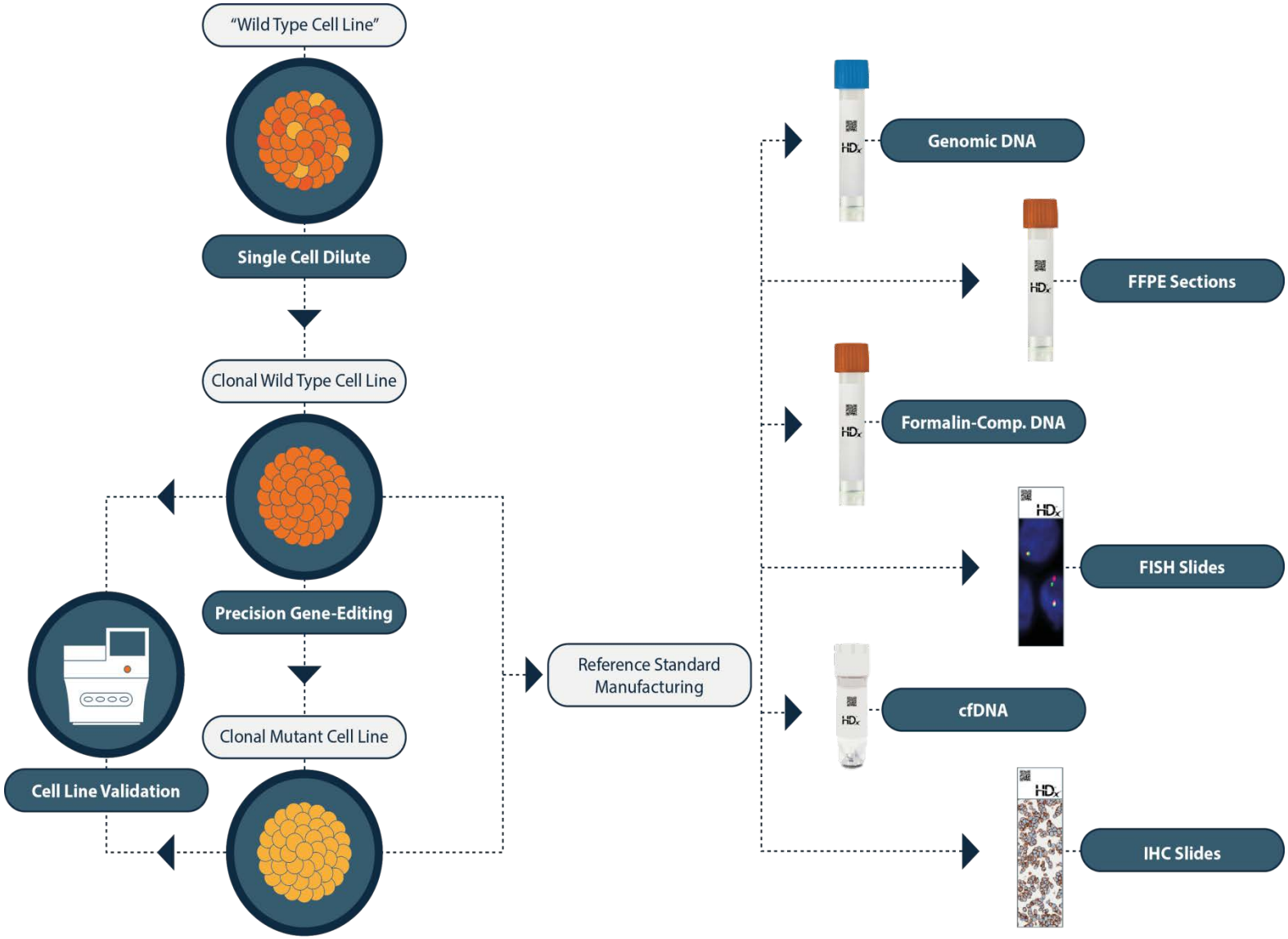
HDx™

Reference Standards allow routine monitoring of the performance of assays, platforms, workflows and operators



Sources of variability within a standard molecular assay workflow

Engineering Cell Lines into Reference Standards



Advantages of Engineering Cell Lines into Reference Materials

Mimics individual patient genetics

Variants presented in relevant genomic context

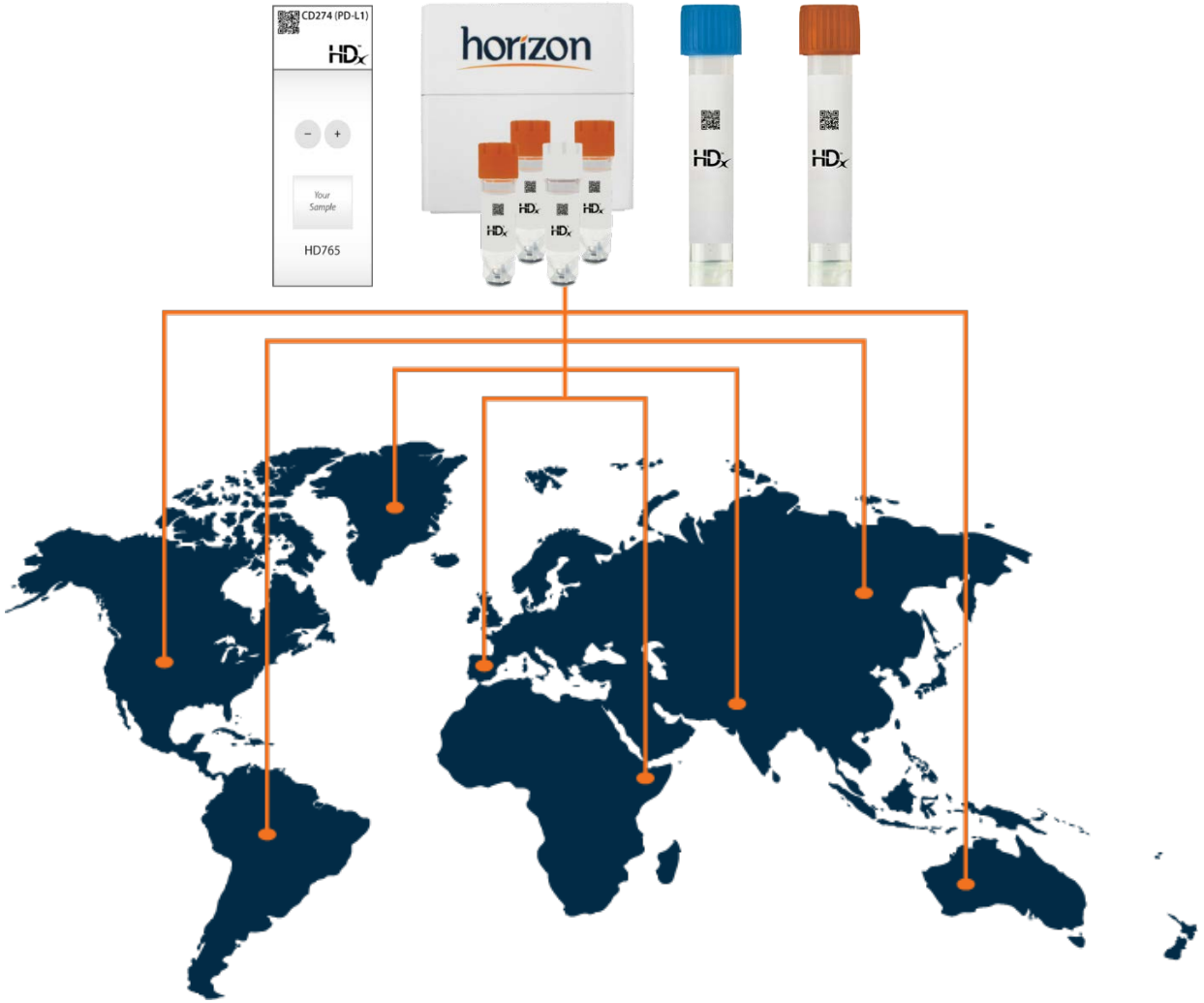
Range of allelic frequencies

Quality-controlled and validated

Prepared under a certified quality management system

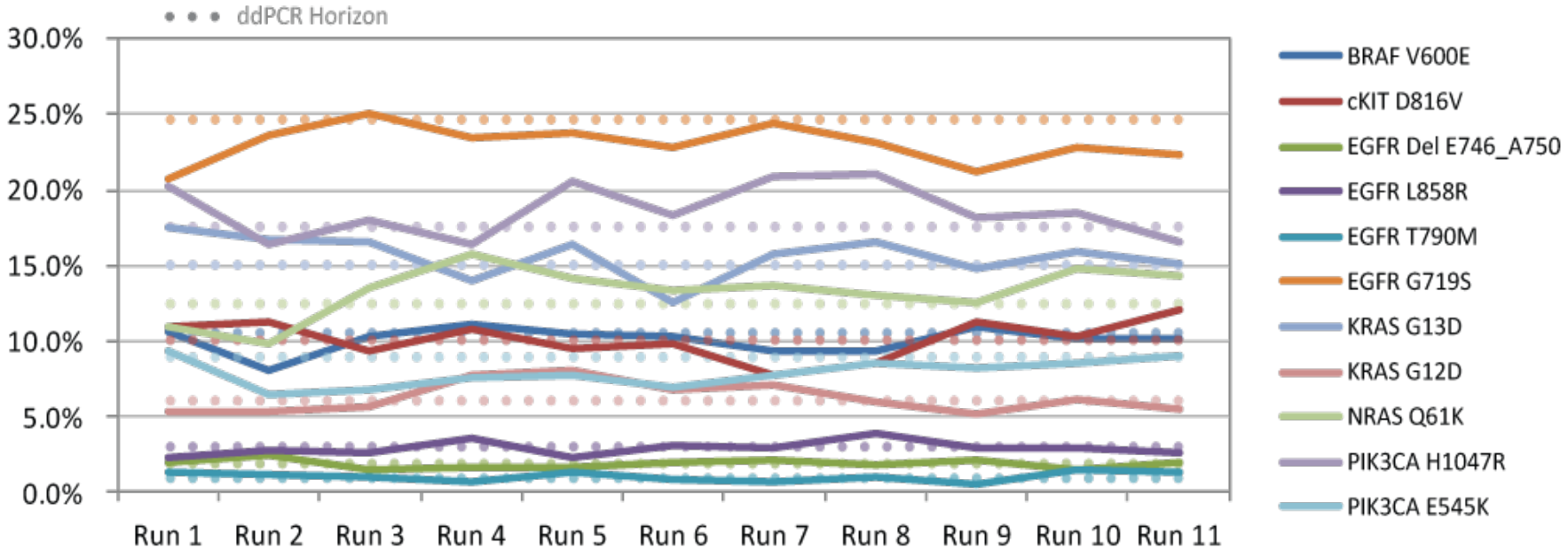
Renewable and affordable

Quality Manufacturing for Widespread Use

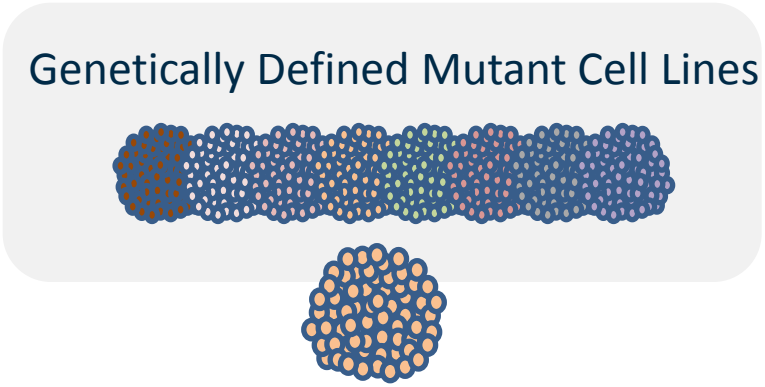


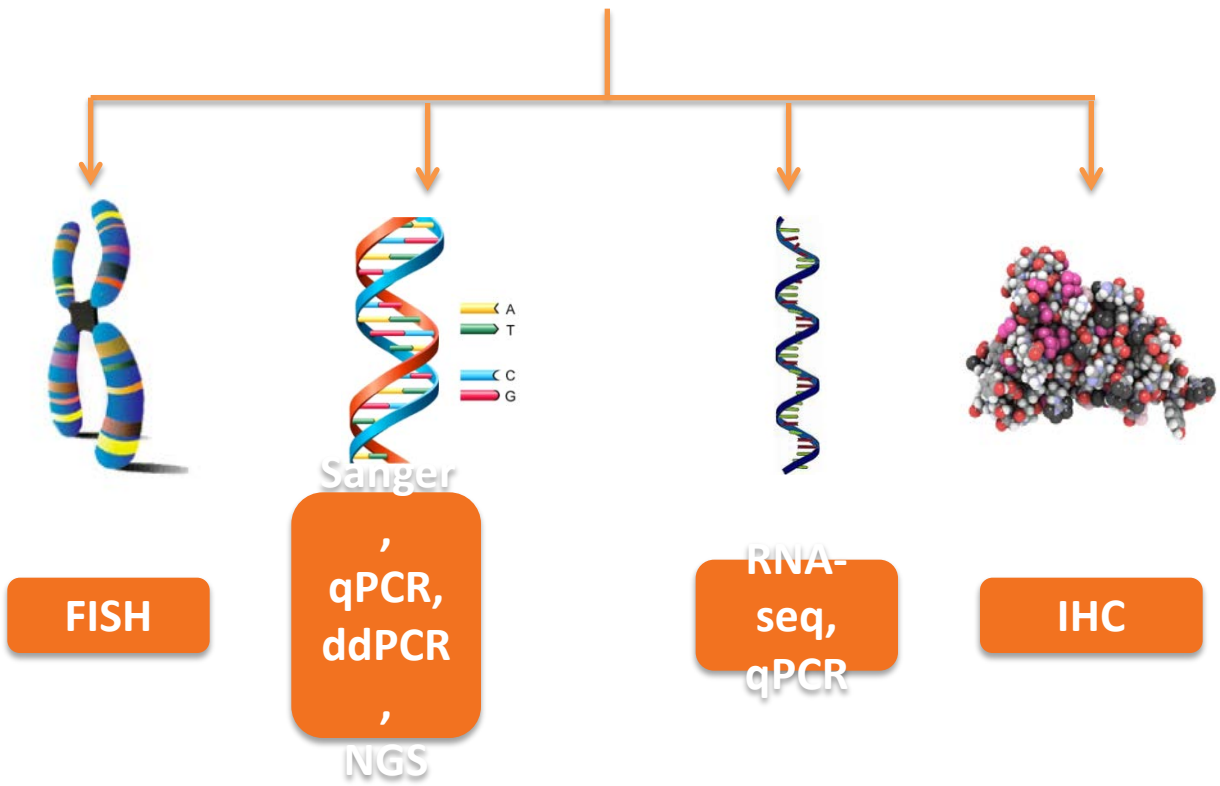
Routine Assay Monitoring

Results from a commercially-available NGS-based Oncology Panel evaluated using Horizon's Quantitative Multiplex Reference Standard (QMRS) in FFPE format; collected over a period of 8 months.



Developing a Universal Reference Standard





Formats Available:	
Genomic DNA	✓
FFPE sections	✓
RNA	✓
Formalin-Compromised DNA	✓
Cell-Free DNA	✓
FFPE on slides (FISH/IHC)	✓

Horizon's Goal:
Engage with assay developers, clinicians, and regulatory agencies to ensure reference materials are available and fit for purpose.

Your Horizon Contact:

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Product Manager, NGS Products

n.lafranzo@horizondiscovery.com

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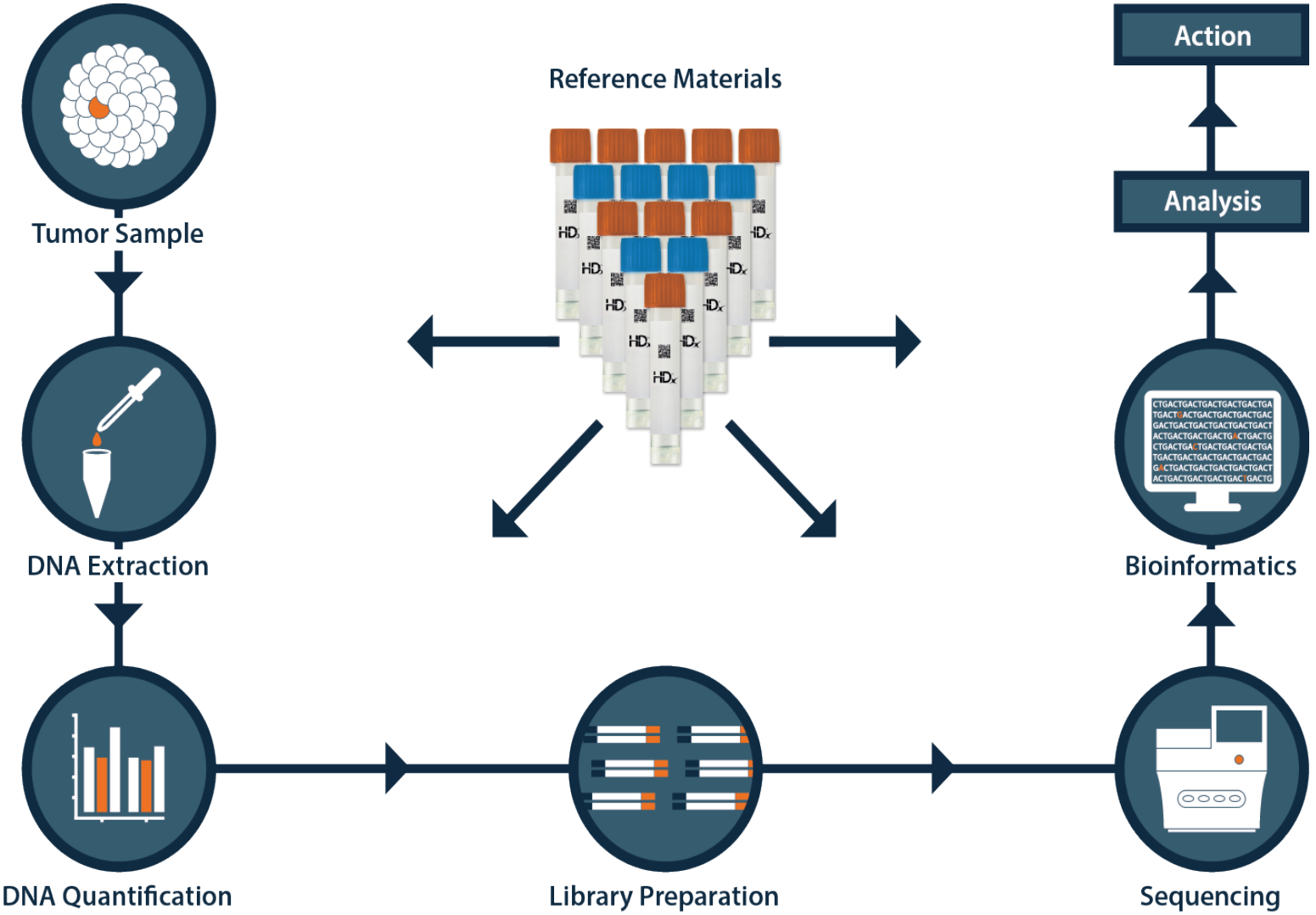
Sources of Variability in the Next Generation Sequencing Workflow



Public Comment Speaker #8

Daryl Pritchard, Ph.D.
Personalized Medicine Coalition

Summary and Wrap-up

Yun-Fu Hu, Ph.D. and

Reena Philip, Ph.D.

Division of Molecular Genetics and Pathology

FDA/CDRH/OIR

Thank You!

Next Generation Sequencing-Based Oncology Panels Workshop

White Oak, MD February 25, 2016